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***On the cover*:** The figures showed the histological changes of spinal cord injury at different time under light microscope. One to forty-eight hours after injury, the hematoma, contusion and subarachnoid hemorrhage (A – C), edema and infiltrated inflammatory cell (E, F) appeared. Four weeks later, scarcicatricial constriction and cavitas formed at the injury site (G, H, asterisk). Multiple contusion could be seen in the gray matter of cephal and caudal end of force spot along the axis of ordinates (I, arrow) and small hemorrhagic lesion (D, arrow). See *Establishment of an acute ventral closed spinal cord injury model* by Hu Wei *et al*, page 18 – 26 in this issue.

Study of several factors affecting on preparation of mouse embryonic stem cells[☆]

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Abstract

The several factors were studied such as the producing methods of embryos, the development stages of embryos and the culture media in order to provide some academic and practical data for the preparation of mouse embryonic stem (ES) cells. Fibroblasts were acquired from 13.5 d embryos of KM mouse. The feeder layer was made by fibroblasts of passage treated with mitomycin C. 3.5-day embryos were cultured on the feeder layer for 4 – 5 days, ICM was separated from the embryonic cells, which was in a good growth status, obvious eminence and without morphological differentiation. The first generation ICM was separated into small pieces of cell masse and then was cultured on the feeder layer in ES culture medium. The passages were carried out according to the appearance of new ES colonies, which grew well and had no differentiation. ICM and different generation of ES cell were identified by morphological observation and alkaline phosphatase staining. The results were showed as following: development ability of embryos from natural mating was better than from superovulation, blastulas had more developmental potential than morulas in the establishment of ES cell lines. DMEM with both low sucrose and high sucrose could be used to culture ES cell, and the medium supplemented with 15% serum was better in culture of ES cell than 10% and 20% group. [Life Science Journal. 2009; 6(1): 1 – 4] (ISSN: 1097 – 8135).

Keywords: mouse; embryonic stem cell; *in vitro* culture

1 Introduction

Mouse embryonic stem cell lines have been established since 1981 and embryonic stem (ES) cells of many animals were isolated successively, such as golden hamster, marten, pig, chiken and monkey. ES cells provided extensive space for study of cell differentiation, animal development, establishment of study model and interpreting gene function (Kagnew *et al*, 2007; Kunarso *et al*, 2008). The premise and basis of various study was establishing and maintaining stem cell lines with differentiation potentiality and normal diploid karyotype (Huang *et al*, 2007). However, there was a low ratio in successful ES cell line's establishment. In this experiment with Kunming mice as experimental animal, the several influencing factors on isolation and culture of ES cells were studied in order to provide theoretical and practical basis for establishment of stable and efficient system of isolation and culture of ES cells.

2 Materials and Methods

2.1 Experimental animals

Kunming strain mice (6 – 8 weeks) were offered by Medicine Factory of Heilongjiang.

2.2 Major reagent

The major reagents were trypsin (1 : 250, Amresco), fetal bovine serum (FBS, Gibco), mitomycin C (Sigma), PMSG (Ningbo hormone factory), HCG (Ningbo hormone factory), ethylenediamine tetraacetic acid (EDTA, Amresco).

The culture media were as follows:

Embryo culture medium I: DMEM (high sucrose)

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+ 15% serum + 2 mmol/L L-glutaminate + 100 IU/ml penicillin + 100 μg/ml streptomycin.

Embryo culture medium II: DMEM (high sucrose) + 20% serum + 0.1 mmol/L β-mercaptoethanol + 2 mmol/L L-glutaminate + 1000 IU/ml LIF + 100 IU/ml penicillin + 100 μg/ml streptomycin.

Embryo culture medium III: DMEM (low sucrose) + 15% serum + 0.1 mmol/L β-mercaptoethanol + 2 mmol/L L-glutaminate + 1000 IU/ml LIF + 100 IU/ml penicillin + 100 μg/ml streptomycin.

2.3 Method

Fibroblast was acquired from 13.5-day embryos of Kunming mice. The feeder layer was made by fibroblast treated with mitomycin C (Lang *et al*, 2006). Mice were superovulated with 10 IU of PMSG and 10 IU of hCG, the embryos of 3.5 days after mating were flushed out from uterus in sterile environment, transferred into inactivated MEF dish and cultured for 4 – 5 days in 5% CO_2, 37 ºC. ICM colonies with well growing status, obvious eminence and without morphological differentiation were passaged. ICM were collected by glass pipette, washed in PBS, incubated individually in the drops of digestive enzyme buffer for 1 – 5 minutes and were dispersed into small pieces of mass by glass pipette. Then they were transferred into MEF dishes and cultured under 5% CO_2, 37 ºC. Observation was taken everyday. Passages were carried out according to the appearance of new ES colonies which grew well and had not differentiated. ES cells were identified by means of alkaline phosphatase staining.

2.4 Statistical analysis

All data were analyzed by SPSS, the value less than 0.05 was considered to be significantly different.

3 Results

3.1 Culture of embryos (Table 1)

Table 1. Comparison of culture of embryos between the natural mating and superovulation (n = 6)

Method of acquiring ovum	Number of embryos	Ratio of attaching wall cultured 3d (%)	Ratio of ICM growing (%)	Ratio of growth ICM after passage (%)
natural mating	60	90.0 ± 3.65[a]	81.66 ± 3.07[a]	50.0 ± 4.83[a]
superovulation	60	83.33 ± 2.11[a]	73.33 ± 3.33[a]	35.0 ± 2.24[b]

[a,b] meant that there was statistical difference between them (P < 0.05). n: number of repetition.

3.2 Comparison of attachment ratio of morula and blastula, ICM growing ratio and ratio of growth ICM after passage (Table 2)

Table 2. Comparison of attachment ratio of morula and blastula, ICM growing ratio and ratio of growth ICM after passage (n = 15)

Categories of embryo	Number of embryo	Ratio of attaching wall cultured 72 h (%)	Ratio of ICM growing cultured 96 h (%)	Ratio of growth ICM after passage cultured 96 h (%)
Morula	164	38.49 ± 2.46[a]	37.16 ± 2.57[a]	27.40 ± 2.01[a]
Blastula	149	80.34 ± 3.11[b]	73.45 ± 3.92[b]	40.50 ± 3.02[b]

[a,b] meant that there was statistical difference between them (P < 0.05). n: number of repetition.

3.3 Effect of different culture medium on embryo culture (Table 3)

Table 3. Result of embryo culture in different culture medium (n = 6)

Culture medium	Numbers of blastula	Ratio of attaching embryo cultured 72 h (%)	Ratio of ICM growing cultured 96 h (%)	Ratio of growth ICM after passage cultured 96 h (%)
I	89	80.34 ± 3.11[a]	73.45 ± 3.92[a]	40.50 ± 3.02[a]
II	71	79.50 ± 5.61[a]	71.79 ± 5.0[a]	44.33 ± 5.60[a]
III	75	60.13 ± 4.03[b]	48.44 ± 4.93[b]	26.54 ± 6.24[b]
IV	69	55.32 ± 5.98[b]	43.65 ± 6.05[b]	39.55 ± 6.81[a]

[a,b] meant that there was statistical difference between them (P < 0.05). n: number of repetition.

3.4 Effect of ES cell culture with different methods (Table 4)

3.5 Growth and identification of ES cells

24 hours after being cultured in MEF dish, small ICM mass attached to the wall and proliferated successfully, and various differentiated cells were observed 2 – 3 days later. It was generally about 4 – 6 days that typical ES cell colonies began to appear (Figure 1). Meanwhile, typical ES cell colonies were dispersed and passaged. ICM, ES colony cells and differentiated cells were identified by staining with AKP. The result showed that well growing ICM were strong AKP positive with black color colonies. Trophoblast-like cells and epithelium were AKP negative and light yellow or achromatic color (Figure 2), the fourth generation cells were strong positive and the colonies were black (Figure 3), and fully differentiated ICM formed into trophoblast-like cells, epithelium-like cells and fibroblasts, which were AKP

Table 4. Result of ES cell culture in different methods (n = 6)

Culture medium	Number	Generation (%)				
		1st	2nd	3rd	4th	5th
I	30	20 (49.26 ± 2.77[a])	6 (20.0%)	2 (6.67%)	1 (3.33%)	1 (3.33%)
II	36	13 (35.75 ± 2.67[b])	4 (11.11%)	1 (2.78%)		
III	40	14 (34.48 ± 2.16[b])	4 (10.0%)			
IV	27	15 (48.19 ± 5.14[a])	6 (22.22%)	2 (7.41%)	2 (7.41%)	1 (3.70%)

[a,b] meant that there was statistical difference between them ($P < 0.05$); n: number of repetition; ES cell ratio: (number of ICM forming ES cell colony/number of incipient) × 100%.

negative and light yellow or achromatic color (Figure 4).

4 Analysis and Discussion

4.1 Nature mating and superovulation

There were no significant difference in wall-attaching ratio of 72 h culture and ICM forming ratio between natural mating and superovulation, but there was significant difference in ratio of ICM which could be passaged. It was concluded that embryos of nature mating was better for isolation and clone of mouse ES cells than superovulation. Maybe it was due to high concentration hormone in blood stream affecting uterine environment.

4.2 Morulae and blastulae

ES cells could be isolated from morula and blastula. The study result were different in the establishment of ES cell lines (Bryja *et al*, 2006). Comparing the effects of morulae and blastulae on establishment of ES cell lines during dispersing morulae into single blastomeres which were cultured in MEF to forme cell colonies. Eistetter *et al* (1989) found that morulae were better than blastulae on the establishment of ES cell lines. In this study which routine culture method was used, the attachment ratio of morulae in 72 hours culture was low (38.49 ± 2.46), but most of blastulae attached to the wall in 72 cultured (80.34 ± 3.11) and others degenerated gradually. Morulae developed into blastula and hatched blastocysts which attached to the wall later. If culture condition and other factors were not good for the development of morula, the number of hatched blastocysts and expanded blastocysts decreased, the hatching ratio of embryos and ratio of ICM formation were low and the number of isolated ES cell was poor. The results showed that blastulae were better than morulae in the isolation of ES cell.

4.3 Culture medium

Compared with the high sugar culture medium, the low sugar culture medium produced the low speed of embryo development and the less number of ICM growth. The low speed of ICM differentiation was advantageous to passage and digestion in time, therefor number of growth ICM after passage was not very less (44.33 ± 5.60 *vs*. 39.55 ± 6.81). Glutaminate was important energy substance for embryo developmen. β-mercaptoethano promoted embryonic cells division growth and protected intracellular enzyme and sulfhydryl groups of protein from oxygenization. The results showed that culture medium with 0.1 mmol/β-mercaptoethano have no significant influencing on embryo devepopment. Low sucrose DMEM was suitable to culture observation of ES cells, and the high sugar culture medium was suitable to acquiring ES cells.

DMEM containing serum was a basic culture medium, but there was difference in culture medium with different serum. The serum was natural medium which provided nutritional ingredient to cells, promoted DNA synthesis and contained some growth regulatory factors for cells to grow and propagate. Also serum provided proteinase inhibitors to cells to protect itself from the damage of proteinase of dead cell. When the concentration of serum was increased in culture medium, the speed of cell growth increased, but high concentration of serum was not always suitable to ES cell culture. The medium supplemented with 15% serum was better for culture of ES cell than 10% and 20% group.

In the culture process of ES cell, there was no significant difference between low sucrose and high sucrose DMEM, but comparing to high sucrose DMEM, ES cell colonies grew slowly in low sucrose DMEM with the small size of ES clones and later differentiation ES.

4.4 Identification of ES cells

The activity of alkaline phosphatase in early ES cells was high. Undifferentiated ES cells had typical cloned configuration which were strong AKP positive. Once ES cells began to differentiated, their AKP became negative (Thomson *et al*, 1998). In this study, ES cell colony was

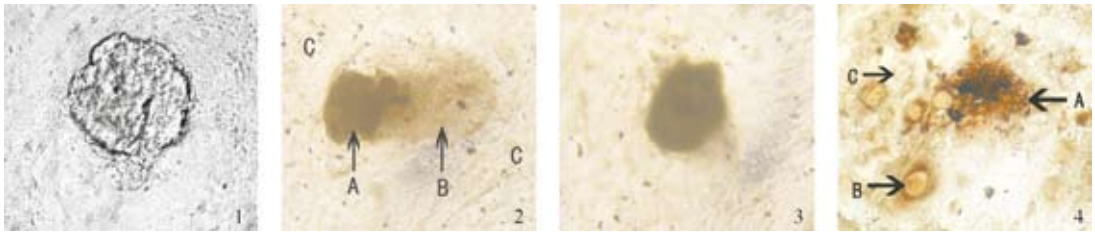

Figure 1. Identification of ES cells. Figure 1.1: ES cell clone, × 100; Figure 1.2: staining of ICM, arrow A shows ICM in black, arrow B points to trophoblast-like cells in light yellow and MEF was achromatic as marked in c area, × 100; Figure 1.3: Clone at passage 4 shows strong AKP positive, × 100; Figure 1.4: Staining of differentiated ICM, arrow A points to trophoblast that was weakly positive and light black, arrow B points to giant cell (epithelioid-like cells), both of fibroblast (c) and giant cell were weakly positive and light yellow, × 100.

strong AKP positive, which meant the ES cells were not differentiated.

5 Conclusion

Development ability of embryoes of natural mating was better than superovulation, blastulae had more developmental potential than morulae in the establishment of ES cell lines. Both low sucrose and high sucrose DMEM could be used to culture ES cells. The medium supplemented with 15% serum was better for culture of ES cell than 10% and 20% group.

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Immunogenicity of lyophilized recombinant adenovirus-based vaccine expressing HIV-1 gagpol in mice[☆]

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Abstract

objective. To evaluate the stability and immunogenicity of lyophilized recombinant adenovirus-based vaccine expressing HIV-1 gagpol(Ad-gagpol vaccine). *Methods*. Screening the optimal novel protector excipient according to the appearance, virus titer and thermostability of the lyophilized Ad-gagpol vaccine. Western blot analysis and IFN-γ Elispot assay were used to detect the immunogenicity of lyophilized Ad-gagpol vaccine in mice. *Results*. Optimal protector excipient and buffer system of lyophilized Ad -based vaccine were identified. It was found to be fairly stable following lengthy exposure to higher temperature. The mice which administered lyophilized Ad-gagpol vaccine produced indistinguishable antibody titer and IFN-γ ELISPOT level compared with liquid Ad-gagpol vaccine group ($P > 0.05$). *Conclusion*. Lyophilized Ad-gagpol vaccine can induce high immunogenicity in mice. The protector containing human serum albumin, trehalose,mannitol, dextran and sucrose was suitable for lyophilized Ad-gagpol vaccine. [Life Science Journal. 2009; 6(1): 13 – 17] (ISSN: 1097 – 8135).

Keywords: Ad-gagpol vaccine; lyophilize; stability; immunogenicity

1 Introduction

Recombinant replication-defective adenoviral (Ad) vectors are being developed as vaccine vehicles to immunize against a number of pathogens[1]. These vectors are promising because they generate strong transgene-specific CD8+ T cell responses in both animal models and people[2]. However, recombinant adenovirus is sensitive to repeat freeze-thaw cycle and easy to lose activity. The rapid loss of vector infectivity during storage and shipment has been reported[3].

In this report, through screening (Data not shown), we identified right protectant excipient, buffer systems and stability of lyophilized recombinant adenovirus-based hiv vaccine expressing HIV-1 gagpol (Ad-gagpol vaccine) during storage at both refrigerated and clinically relevant storage temperatures. Also, evaluate the immunogenicity of lyophilized Ad-gagpol vaccine in mice. We expect that our results can contribute to open further delivery applications for vaccination strategies.

2 Materials and Methods

2.1 Cell culture

HEK293 cells were cultured in Minimum Essential Medium (MEM) supplemented with 5% (v/v) fetal bovine serum (FBS) and 2 mM glutamine. The cells were kept in a humidified atmosphere of 5% CO_2 in air at 37 ºC.

2.2 Preparation of liquid Ad-gagpol vaccine

Ad-gagpol vaccine was amplified in the HEK293 cells. The vaccine was purified from cell lysates by banding twice on CsCl gradients, followed by desalting with PD-10 Desalting columns (USA) equilibrated with sterile 10 mM Tris and 2 mM $MgCl_2$. The virus was resuspended in a PBS solution to obtain liquid Ad-gagpol vaccine.

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2.3 Measuring the crystallization and thawing characteristic of vaccine protector

Consist of 2% trehalose, 1% mannitol, 2% dextran, 0.3% albumin and 2% sucrose (w/v) in 0.01 M sodium phosphate buffer at pH 7.4 were used for this study. Crystallization was measured by registration of the resistance of the stabilizers during the freeze-thawing process. The cool-down speed was 1 ºC/minute, thawing speed was 3 ºC/minute. Change in resistance values for vaccine stabilizer is given in Figure 1.

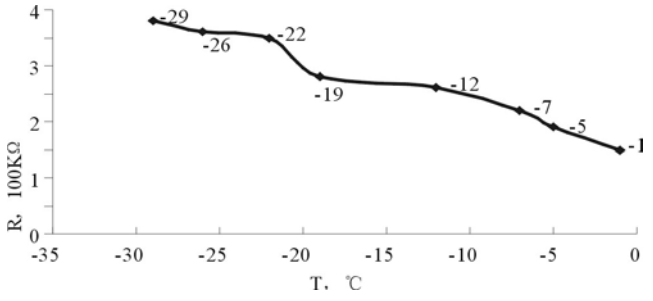

Figure 1. Measurement of the resistance of Ad-gagpol vaccine with protector during freezing and thawing treatment.

2.4 Lyophilization

Lyophilization was carried out using shanghai LYO-0.5 freeze-drier. Equal volumes of the vaccine and protector were mixed. 0.5ml of the mixture was dispensed in sterile small vial . The vaccine vials were pre-frozen at − 40ºC for 3 hours and subjected to the first lyophilization at − 30 ºC for 10 hours and the second lyophilization at 28 ºC for 5 hours at a vacuum of 0.06 m bar. Batches of vaccine containing different stabilizer formulations were lyophilized simultaneously under identical conditions to compare the quality in terms of residual moisture and titre loss during lyophilization.

2.5 Analysis of thermostability of lyophilized vaccine

Lyophilized vaccine vials were exposed at 4 ºC and 37 ºC respectively. Samples were taken from the refrigerator at 2, 6, 10, 13, 17, 21 months; from 37 ºC at 1, 2, 3 and 4 weeks. Exposed samples were reconstituted with 0.5 ml of distilled water and titrated in HEK293 cells. For each time point, ten samples were titrated and their average log10 titer was calculated.

2.6 Measurement of titers of adenoviruses

HEK293 cells were seeded in 96-well plates at 5×10^3 cells/well. Twenty-four hours later, the medium was replaced with 50µl of medium that contained ten-fold serial dilutions of the adenovirus used, from 1×10^2 to 1×10^{-11}. The plates were centrifuged at 1000 × g for 10 min and then placed in an incubator. For determination of the CPE, the plates were incubated for a minimum of 10 days, during which time there was no evidence of a CPE in the control wells. Plates were examined daily for the CPE of the virus on the infected cells and the titer was calculated.

2.7 Mice and immunization schedule

Female BALB/c mice were divided into the liquid vaccination group, the lyophilized vaccination group, and the control group. The mice of liquid vaccination group and lyophilized vaccination group were administered a single intramuscular injection with 5×10^8 pfu Ad-gagpol vaccine. Control group mice were inoculated by PBS.

2.8 Antibody detection

Collect MoltIII B cell (stably expresses the HIV-protein cell line) supernatant, then centrifuge at 26000 rpm/minute. Resuspend the precipitation and run SDS-PAGE, transfer to cellulose membrane as antigen detecting mouse blood serum. The blocked membranes were placed in a multiscreen apparatus (Bio-Rad, USA), and approximately 100 µl of diluted serum was pipetted into individual lanes. Serum samples were diluted 1 : 50 with 3% milk-PBS. Following a 2-hour incubation at RT, the blots were removed from the apparatus and washed three times in T-PBS. The membranes were then incubated 1 hour at RT with antimouse IgG antibodies conjugated with AP and washed three times with T-PBS. The blots were visualized with NBT and BCIP in AP buffer (Sigma, USA), as recommended by the manufacturer. The blots were developed by using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

2.9 IFN-γ ELISPOT assay

An ELISPOT assay kits (USA) was used to determine vaccine elicited IFN-γ responses in BALB/c mice. Spleen lymphocytes from the immunized mice were cultured in a plate with medium. 96-well plates were coated with purified anti-mouse IFN-γ monoclonal antibodies, and incubated at 4 ºC overnight. Mice splenocytes were isolated and red blood cells were lysed by RBC lysis buffer. Cells were washed two times and re-suspended in complete culture medium. After counting, splenocytes were then adjusted to the concentration of 4×10^6 cells/ml and plated into pre-coated 96-well Elispot plate at 100 µl/well with addition of 100 µl peptide

P7G (AMQMLKETI, 1 μg/ml). The Elispot plates were incubated and developed according to the kit instruction. Finally, plates were air-dried and the resulting spots were counted with Immunospot Reader (USA). Peptide specific IFN-γ Elispot responses were considered as positive only when the responses were 4-fold above negative control with no peptide stimulation.

3 Results

3.1 Physical characters

Appearance of the lyophilized Ad-gagpol vaccine still keep good, white and loose at 37 ºC for 2 months. Furthermore, it can be reconstituted rapidly with distilled water.

3.2 Crystallization and thawing temperatures of stabilized Ad-gagpol vaccines

Freeze and thawing of the Ad-gagpol vaccine and protectors clearly showed that only at low temperatures the products are completely frozen (Figure 2). Parts of the products crystallized between 0 ºC and − 19 ºC, whereas the main part crystallized up to a temperature of − 19 ºC. The Eutectic point temperature with a minimum range in the resistance value is reached below − 22 °C. The thawing curve mirrors in general the crystallization curve of the products.

Figure 2. Shape of lyophilized Ad-gagpol vaccine stored at the 60st day at 37 ºC.

3.3 Thermostability of lyophilized Ad-gagpol vaccine

Virus titration was carried out to determine the infectivity of the freeze-dried vaccine after exposure at 4 ºC and 37 ºC for different time intervals. The infectivity titres thus obtained were subjected to regression analysis. Table 1 and Table 2 summarize the results of regression analysis at different temperatures. It could be possible to calculate half-life (time required for loss of half the original titre, i.e. 0.30 $\log_{10}$ PFU based on the degradation constant) by assaying more number of samples over a long period beyond.

The titer of Ad-gagpol vaccine before and after lyophilized decreased by 0.17 $\log_{10}$ (Table 3). As for lyophilized vaccine, 0.50 $\log_{10}$ and 0.60 $\log_{10}$ titer reduced after heating at 37 ºC for 14 days and 21 days, respectively. Its half-life is 11.07 days at 37 ºC. The loss was very small.While as for liquid vaccine, the half-life is 1.03 days. 2.50 $\log_{10}$ titer reduced after heating at 37 ºC for 7 days (Table 1, Figure 3). We also assess long-term stability of lyophilized Ad-gagpol vaccine stored at 4 ºC, a drop in titer of approximately 0.6Log_{10}PFU/ml for 17 months, but the titer of the liquid vaccine drop apparently 1.6Log_{10}PFU/ml after stored for 2 months (Table 2, Figure 4). These data showed lyophilized vaccine viruses were found to be fairly stable following lengthy exposure to higher temperature, compared with the current liquid vaccine preparations.

Table 1. Comparison of degradation values of lyophilized and liquid Ad-gagpol vaccine at 37 ºC

Temperature (ºC)	Stabilizer	Initial titer	Regression equation	Half-life (days)
37	lyophilized	7.6	y = – 0.0271x + 7.54	11.07
37	liquid	8.2	y = – 0.2914x + 8.0467	1.03

Table 2. Comparison of degradation values of lyophilized and liquid Ad-gagpol vaccine at 4ºC

Temperature (ºC)	Stabilizer	Initial titer	Regression equation	Half-life (months)
4	lyophilized	7.6	y = – 0.0281x + 7.4683	10.68
4	liquid	7.8	y = – 0.3181x + 7.3157	0.94

Table 3. Virus titers of Ad-gagpol vaccine before and after lyophilization

Group	Titer (Log_{10} PFU/ml)		
	Before lyophilization	After lyophilization	Loss
lyophilized	7.43	7.26	0.17
Liquid	8.30	6.80	1.50

3.4 Humoral immune responses

The mouse blood serum according to 1 : 50 dilution, anti-P24 antibody was used to detect humoral immune

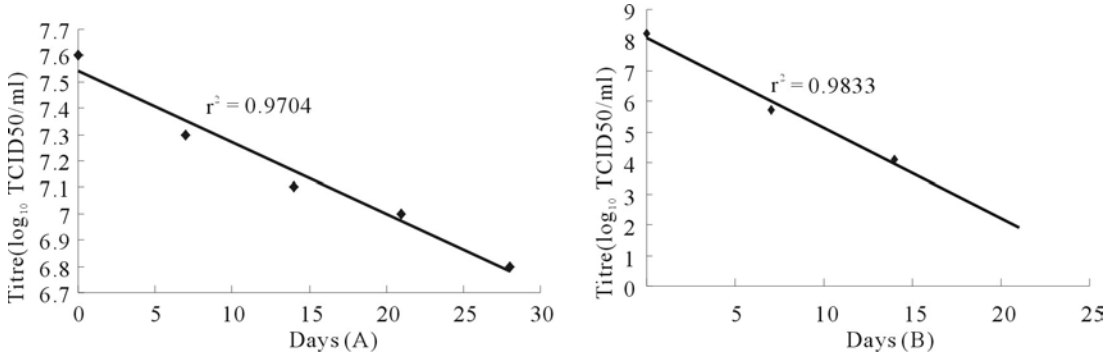

Figure 3. Degradation curves (37 ºC) for lyophilized and liquid Ad-gagpol vaccine. A: ophilized Ad-gagpol vaccine B: liquid Ad-gagpol vaccine.

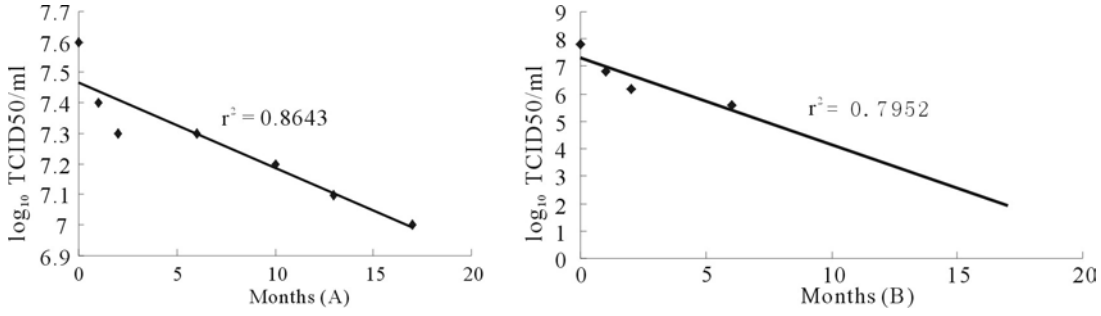

Figure 4. Degradation curves (4º C) for lyophilized and liquid Ad-gagpol vaccine. A: lyophilized Ad-gagpol vaccine B: liquid Ad-gagpol vaccine.

response in blood serum with Western blot law. Figure 5 demonstrates that two vaccines groups produce the same antibody level.

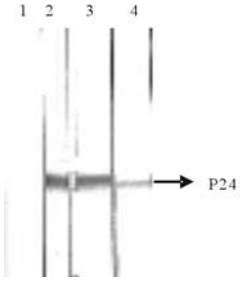

Figure 5. Western blot analysis the antibody level of lyophilized and liquid Ad-gagpol vaccine immunized mice. 1: PBS negative control, 2: lyophilized Ad-gagpol vaccine, 3: liquid Ad-gagpol vaccine, 4: mice P24 Ab positive control.

3.5 IFN-γ ELISPOT

Ag-specific CD8 T cells were analyzed by IFN-γ ELISPOT. The evaluated results for IFN-γ production are expressed as the mean numbers of IFN-γ secreting cells (spots) per 10^5 splenocytes. The number of IFN-γ-secreting lymphocytes elicited by lyophilized vaccine and liquid vaccine in mice was approximated (275 versus 238, $P > 0.05$, Figure 6). These results illustrate that the mice which administered lyophilized Ad-gagpol vaccine produced indistinguishable IFN-γ ELISPOT level compared with liquid Ad-gagpol vaccine group.

4 Discussion

Adenovirus is significantly inactivated at pH values below 6, and undergoes capsid degradation during repeated freeze-thaw cycles[4]. Scientists generally place their novel vectors in buffer, add glycerol and store the preparation at − 80 ºC. Vectors stored under these conditions must be quickly shipped to remote sites on dry ice,which is somewhat costly[5].This formulation also requires extensive dilution before administration to reduce the toxicity of glycerol.

Although freeze-dry is a common method in preserving live biological samples, it usually leads to protein denaturation and drop in cell viability. Thus, selecting the optimal protector for the lyophilization process of the vaccine is a critical step in vaccine

production. This is directly related to the infective titer and stability of the vaccine. Surface adsorption, freeze-thaw and free-radical oxidation are the major inactivation pathways for Ad during storage[6]. The noncovalent interactions of viral structures are sensitive to variations in pH, temperature, and composition of the surrounding environment, particularly to osmotic stress[7].

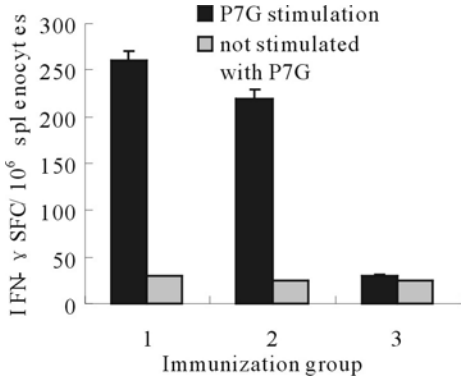

Figure 6. Cell-mediated immune response by ELISPOT. 1: lyophilized Ad-gagpol vaccine; 2: liquid Ad-gagpol vaccine; 3: PBS negative control.

Through screening, the protector containing human serum albumin, trehalose, mannitol, dextran and sucrose showed good protective effect on lyophilized adenovirus-based live vaccine.

Carbohydrate and polyalcohol enhance the hydrophobic interaction with protein through influencing the water molecule conformation, thus preventing heat denaturation and enhanced stability for proteins in solution. When water is removed during drying, the protectors can hydrogen bond with the protein as water does, thereby preserving the native structure during processing by thermodynamic stabilization of the native conformation. Stabilization during storage is then a result of preservation of the native structure in the solid state , regardless of the mechanism responsible for such preservation[8]. Furthermore, trehalose and dextran inhibit potential for virus adsorption and aggregation. The free-radical oxidation inhibitor mannitol was determined to be effective stabilizer of virus. The addition of 1% albumin prevented viral aggregation and allowed the purified virus to retain its activity after filter sterilization. Furthermore, viral activity was retained within the 1% albumin solution for at least 1 week at 37 ºC and for 2 weeks at 4 ºC, whereas viral activity within the albumin-free solution was quickly lost[9].

According to the results on the appearance, infective titer, accelerated thermal stability and long term stability tests, combination of trehalose, mannitol, dextran, albumin and sucrose makes a suitable protector compound. At the same time, it is pharmaceutical acceptable and convenient for large-scale production of the vaccine.

Excipients employed in vector formulations must be suitable for use *in vivo*. To determine the potentiality of trehalose, mannitol, dextran, albumin and sucrose cause a loss of immunogenicity for lyophilized Ad-gagpol vaccine, The mice of liquid vaccination group and lyophilized vaccination group were administered a single intramuscular injection with 5×10^8 pfu Ad-gagpol vaccine.The IFN-γ ELISPOT assay and immunoblot assay were used to monitor the cellular and humoral immune responses. The results (Figure5, Figure6) indicated that the immunogenicity of lyophilized Ad-gagpol vaccine and liquid Ad-gagpol vaccine was indisti nguishable,showing that trehalose, mannitol, dextran and inositol did not significantly alter the immunogenicity of the vaccine.

These results demonstrate that lyophilized Ad-gagpol vaccine is as effective to induce immune response in mice as liquid vaccine. In addition,it can be shipped and stored at the room temperature,supporting its further evaluation and application in clinical studies.

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Effect of cryopreservation on the development and DNA methylation patterns of *Arabidopsis thaliana*☆

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Abstract

Seedlings of *Arabidopsis thaliana* were used to assess its recovery ability, DNA methylation alteration and exchanges after cryopreservation. The survival rate and the level of DNA methylation were evaluated after pre-treatment (loading and cryoprotection), unloading and recovery from cryopreservation of the plants. Over 98% of the plants were survived after pre-treatment and unloading without cryostorage in liquid nitrogen, whereas the survival rate decreased to 93.8% after pre-treatment, cryostorage and then unloading. The results suggest that cryostorage and cryopreservation of seedlings obviously impact on the change of levels in the DNA methylation determined by methylation-sensitive amplified polymorphism technique in *Arabidopsis*. [Life Science Journal. 2009; 6(1): 55 – 60] (ISSN: 1097 – 8135).

Keywords: *Arabidopsis thaliana*; cryopreservation; survival rate; MSAP; DNA methylation

1 Introduction

Among the long-term conservation ways of plant germplasm, cryopreservation is actually the most valuable because it needs very limited space and low maintenance, can be protected from contamination[1]. As a simple alternation method of cryopreservation, vitrification has been successfully applied to plant shoot tips, embrogentic, plasterm and so on[2–5].

The assessment of plant genetic integrity after cryopreservation has been a subject of increasing interest, especially with the fast development of DNA-based techniques[6]. In cryopreservation, plant materials are stored in liquid nitrogen (LN) where both cell division and metabolism are arrested, and the materials can be thus preserved theoretically without genetic alteration for an unlimited period of time. However, various factors associated with cryopreservation and recovery procedures underlie the production of cryo-selection and somaclonal variations[7]. Cryoconservation may usually induce DNA alterations, especially at the epigenetic level accomplished by altered DNA methylation status. Hao *et al* found some demethylated sites after cryopreservation by vitrification in strawberry and apple[8–9].

In eukaryotes and particularly in higher plants, 5-methylcytosine is the predominant modified base[2]. Variation in DNA methylation can lead to alterations in chromatin structure and changes in gene expression. Furthermore disturbance of intrinsic DNA methylation patterns may have structural and functional consequences to the organisms with this epigenetic code[10]. In *Arabidopsis*, DNA methylation levels are critical for embryogenesis, seed viability[11], and drastic global reduction of cytosine methylation due to loss-of-function mutation of the *Met*1 gene (counterpart of the mammalian Dnmt1) or *DDM*1 (decrease in DNA methylation1) gene, albeit non-lethal, produces pleiotropically defective phenotypes and developmental abnormality[12–14].

Two main methods are routinely used for the investigation of DNA methylation in the tissues of the eukaryotic organisms, and bisulfites or methylation-sensitive restriction enzymes are applied. In methylation-sensitive amplified polymorphism (MSAP) analysis, isoschizomers are used to detect the of DNA methylation. The amplified fragment length polymorphism (AFLP) technique was adapted, in which the isoschizomers *Hpa*

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II and *Msp* I are employed as "frequent-cutter" enzymes for AFLP instead of the usual *Mse* I[15]. This method has been proven reliability and used to study DNA methylation of several corps[16–22].

Although many studies have been conducted to test the genetic stability of cryopreserved plant materials, the research using the *Arabidopsis thaliana* seedlings as materials has not been done. In this study, the procedures of vitrification were modified, and high rates of survival had been evaluated. Although, minor alterations of methylation pattterns were observed by MSAP technique after the plants were treated for cryopreservation, the development of all the plants (including those treated with cryoprotection and cryostorage in LN) were normal.

2 Materials and Methods

2.1 Plant material and growth conditions

Arabidopsis seeds (Landsberg) used in the experiments were from Henan Laboratory of Plant Stress Biology. The seeds were sterilized for five minutes with 0.1% mercuric chloride and rinsed with sterile water 4 – 5 times, then sown on Petri plates (9 cm diam) containing solidified MS medium[23] with agar 0.7%. The seeds were kept in 4 ºC for 72 hours, then incubated at 22 ºC under cool-white fluorescent lights, 100 μmol/m^2 with 10 hours light per 14 hours dark photoperiod.

The method was modified by Liu *et al*[24]. Two days old seedlings were immersed in loading solution (MS liquid medium + 2 M glycerol + 0.4 M sucrose) for 20 minutes at room temperature (1.0 ml for 30 – 40 seedlings in one 2 ml cryovial). Loading solution was removed from the cryovial and rapidly replaced by filtered sterilized cryoprotective solution PVS2 (30% w/v glycerol, 15% w/v ethylene glycol and 15% w/v DMSO in liquid MS medium supplemented with 0.4 M sucrose) and left at 0 ºC for 50 minutes. The cryovials were then rapidly immersed in LN for at least 1 hours. The thawing was carried out by immersing cryovials in a water bath at 40 ºC for 1 minute, and cryovials were shaked vigorously. Subsequently, PVS2 solution was unloaded by removing it out from the tube and replacing with unloading solution (MS liquid medium + 1.2 M sucrose) for 40 minutes, and the solution was replaced once 10 minutes. The seedlings were then cultured on MS medium and maintained under the exactly same condition as that for seed germination. The cryopreserved samples had been done with exactly as above steps, some seedlings were treated only with the pre-treatment (loading and cryoprotection), then employed unloading and reculture, which hadn't been cryostoraged in LN and thawed in 40 ºC water bath.

2.2 Plants survival and development

The survival rates were evaluated after 5 days of thawing. Seedlings were transferred into solid culture medium and placed in the same growth chamber after growing on MS about 15 days. Regular management was kept until new seeds were harvested.

2.2.1 DNA extraction. When all the seedlings begin to have stalks, the leaves of about 30 seedlings once treatment (from the same pool) were used to extract DNA using CTAB method. The DNA was used for the following analysis.

2.2.2 MSAP analysis. The MSAP was adopted from Cervera *et al*[25]. Aliquots (250 ng) of DNA were digested for 3 hours at 37 ºC, 3 Units (U) *Eco*R I in 20 μl of 2 μl 10 × buffer (500 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 10 mM Dithiothreitol; 100 mM NaCl). After digestion, DNA was precipitated and digested with 3 U *Hpa* II in 20 μl of 2 μl 10 × buffer (100 mM Tris-HCl, pH7.5; 100 mM $MgCl_2$; 10 mM Dithiothreitol) for 3 hours at 37 ºC. *Eco*R I/*Msp* I DNA digestion were carried out in a final volume of 20 μl with 3 U each of *Eco*R I and *Msp* I, 2 μl 10 × buffer (330 mM Tris-Ac, pH 7.9; 100 mM Mg-Ac; 5 mM Dithiothreitol; 660 mM K-Ac), 4.0 μl BSA and 250 ng of DNA for 3 hours at 37 ºC. The DNA fragments from the two reactions were added separately to an equal volume of the adapter/ligation solution, and the ligation reaction was allowed to proceed at 25 ºC for 2 hours, the ligation mixture was diluted five-fold for use as templates for the first selective amplification with *Eco*R I + A primers and *Hpa* II/*Msp* I + T primers. The PCRs were performed in a 20 μl volume of 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 50 ng of each primer, 0.4 U Taq DNA polymerases and 5.0 μl of diluted fragments. The product was diluted 20-fold and used as the templates for selective amplification reaction. The selective PCR was performed in a final volume of 10 μl following the protocol of Vos *et al*[26]. All the sequences of the adapters and primers used have been list in Table 1. The PCR amplification was carried out using Zou *et al*[27]. The product of selective amplification were denatured by adding a 1/2 volume of fomamide-buffer (98% formamide; 10 mM EDTA, pH8; 0.05% bromophenol and 0.05% xylene cyanol) and heating for 3 minutes at 94 ºC, then moved into 0 ºC immediately. Electrophoresis was performed with aliquots of each sample on 6% polyacrylamide gel (acrylamide/bisacyylamide, 19 : 1) containing 7.5 M urea and 1 × TBE for 3.0 hours at 55 W.

Then the gel was stained with silver[28].

3 Results

The process of cryopreservation comprises three steps: pre-treatment (loading and cryoprotection), freeze-thawing (cryostorage in LN and bath in 40 ºC water), unloading and reculture. The survival rate, stability of DNA methylation were determined.

3.1 Plants survival and development

Seedlings were pre-treated, unloaded (not cryostorage in LN and thawing in water) and then recultured in the conditions described above (see methods). Almost all seedlings (96.8% – 100%) were survived and started to grow up within 5 days. Other seedlings after pre-treatments were followed by cryostorage in LN, then thawing in 40 ºC water, unloading and reculture on MS medium. After cryostorage, the survival ability of seedlings decreased a little, and the average survival rate reached 93.8%. Twenty days after re-culture, all the plants with treatments (involving freeze-thawing or not) did not show morphological alterations as compared with the untreated and unfrozen controls (Figure 1.4), but in the first, seedlings cryostoraged in LN grew more slowly than the controls and those treated without freeze-thawing (Figures 1.1 – 1.3).

3.2 Analysis of the DNA methylation patterns

Hpa II and *Msp* I are isoschizomers frequently used to detect cytosine methylation. Both restriction enzymes recognize the tetranucleotide sequence 5'-CCGG-3'. *Hpa* II is inactive when one of the two cytosine is fully-methylated (both DNA strands are methylated), but it cuts the hemi-methylated 5'-CCGG-3' (only one DNA strand is methylated); whereas, *Msp* I cuts 5'-CmCGG-3', instead of 5'-mCCGG-3'. Therefore, type I bands have been previously associated with unmethylated DNA sequences; while, type II and type III bands have been associated with methylated and hemi-methylated DNA sequences, respectively. The DNA methylation of three samples (1: the control; 2: plants after cryopreservation; 3: plants treated by cryopreservation except freeze-thawing) has been analyzed using MSAP. Three types of MSAP bands were observed. Type I bands were present in both restriction enzyme combinations *Eco*R I/*Msp* I and *Eco*R I/*Hpa* II, while type II bands were present in *Eco*R I/*Hpa* II but absent in *Eco*R I/*Msp* I, and type III MSAP bands were present in *Eco*R I/*Msp* I but absent in *Eco*R I/*Hpa* II (Figure 2). According to Xu *et al*[29], disappearance of type I and type II MSAP bands is mainly due to methylation of the outer cytosine of the 5'-CCGG-3' and 5'-CmCGG-3' sequences, respectively. While appearance of type I and II MSAP bands is largely due to demethylation of the outer cytosine of the 5'-mCCGG-3' and 5'-mCmCGG-3' sequence, respectively. However, appearance and disappearance of type III MSAP bands involve both inner and outer cytosines. Methylation alteration of the inner cytosine is responsible for exchanges between type I and type II bands.

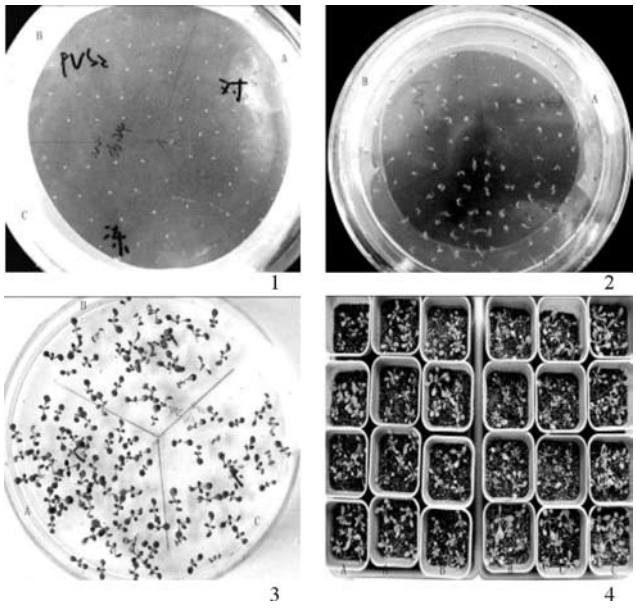

Figure 1. Developmental phenotypes of the plants (A: controls, B: treatment with cryopreservation expect cryostorage in LN and thawing in 40 °C water, C: treatment with the whole process of the cryopreservation). Figure 1.1: seedlings of the day just treatments; Figure 1.2: five days seedling; Figure 1.3: ten days old seedlings; Figure 1.4: seedlings of twenty days old, recultured in the soild from MS.

Sixteen primer combinations (Table 1) were used and a total of 619 distinct and reproducible bands were generated. In order to reveal the change among the three samples, all bands belonging to each of the banding patterns were scored (Table 2). Type I, II and III MSAP bands that were present in three samples were designated MSAP-banding patterns 1, 2, 3, respectively. Sixty-one changed bands were observed among all three samples and exhibited 27 MSAP-banding patterns. Various changes in banding types among the different samples were observed. Among these changes, both de novo methylation and demethylation states in sequence were observed (Table 3). Base on the statistics, alterations in DNA methylation of the controls were significantly

different from the plants treated without cryostorage in LN, while those of controls and treatment with cryopreservation were not significantly. Compared to the control, 50 DNA methylation patterns of the samples treated without LN and water were observed to be changed. 15 sites showed de novo methylation, and 6 sites were demethylated. 33 different patterns were seen between the controls and the samples after cryopreservation. The treatment samples after cryostorage in LN showed 11 sites de novo methylation and 7 sites demethylation contrasted with those without cryostorage in LN.

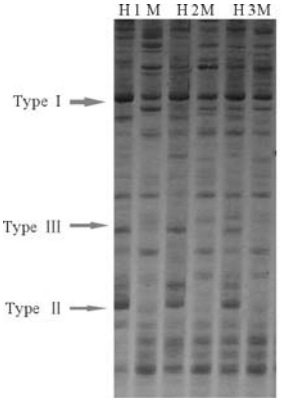

Figure 2. MSAP analysis of plants. DNA fingerprints were generated with the primer combination *Eco*R I + ACA/*Hpa* II + TCAA. H and M refer to digestion with *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I, respectively. 1: control; 2: cryopreservation without cryostorage and thawing; 3: cryopreservation. Arrows lead to the corresponding MSAP- banding patterns.

4 Discussion

4.1 Modification of experiment procedures

In our work, the method of cryopreservation was used from liu *et al*[24]. The thawing step they employed was at room temperature for 5 to 10 minutes, while we have changed it, rapidly warming in 40 ºC water bath for 1 minute. The average survival rate with our method reached 93.8%, this recover percentage is higher than that achieved by Liu *et al*. According to Martin and Gonzalez-Benito[30], rapid warming of plants (at 40 ºC instead at room temperatures) could increase thire recovery rate, and which was further bolstered by our results. Thawing as rapidly as possible could help to avoid the phenomenon of ice recrystallization of seedlings[31]. We found high rate of recovery after cryoprotection, but a low recovery rate after the cryostorage, the possible reason is damage resulting from ice-crystal growth during rewarming. Usually, during the cryopresevation procedure, materials cryostorage in LN at least for 24 hours. To keep the treated seedlings have same times of development with the controls, we have kept the seedlings in LN for only 1 hour. In our repeat experiments, the results showed that the time of cryostorage in LN had no impact on the survival rates (data not show).

During the MSAP analysis, fragment digestion and ligation condition were improved by reducing the DNA and restriction enzyme concentrations while optimizing separate digestion steps and increased the number of amplified fragments and improved fingerprint readability.

4.2 DNA methylation level change

In a MSAP analysis, only *Eco*R I/*Msp* I or *Eco*R I/*Hpa* II fragments were likely to be both amplified and visualized on the gels. *Msp* I and *Hpa* II showed no differences in cutting a nonmethylated 5'-CCGG-3' sequence and therefore generated type I MSAP bands.

Table 1. Sequences of adapters and primers

	*Eco*R I	*Hpa* II/*MSp* I
Adapter 1	5'-CTCGTAGACTGCGTACC-3'	5'-GATCATGAGTCCTGCT-3'
Adapter 2	3'-CTGACGCATGGTTAA-5'	3'-AGTACTCAGGACGAGC-5'
Pre-selective primer	5'-GACTGCGTACCAATTCA-3'	5'-ATCATGAGTCCTGCTCGG-3'
Primer	(E+A)	(HM+T)
Selective primer	5'-GACTGCGTACCAATTCAAC-3'	5'-ATCATGAGTCCTGCTCGGTCAA-3'
	5'-GACTGCGTACCAATTCAAG-3'	5'-ATCATGAGTCCTGCTCGGTCCA-3'
	5'-GACTGCGTACCAATTCACA-3'	
	5'-GACTGCGTACCAATTCACT-3'	
	5'-GACTGCGTACCAATTCACC-3'	
	5'-GACTGCGTACCAATTCACG-3'	
	5'-GACTGCGTACCAATTCAGC-3'	
	5'-GACTGCGTACCAATTCAGG-3'	

However, differential sensitivity occured when the inner cytosine in the 5'-CCGG-3' sequence was methylated. In this case, only *Msp* I can cut the 5'-CmCGG-3' sequence, and results in "standard" *Eco*R I/*Msp* I fragment without (an) internal 5'-CCGG-3' sequences. *Hpa* II could not cut the 5'-CmCGG-3' sequence until it recognized the next non methylated 5'-CCGG-3' sequences.

Based on nucleotide composition and genome complexity, the genome of *Arabidopsis* is expected to contain an average of 45,000 *Eco*R I sites. Since two *Msp* I/*Hpa* II restriction sites should flank each of these *Eco*R I restriction sites, a total of 90,000 *Eco*R I/*Hpa* II or *Eco*R I/*Msp* I fragment could theoretically be detected using different selective nucleotide, although only CCGG sequences that lie close to *Eco*R I restriction sites can be detected. The probability that a methylated CCGG site will be digested by *Hpa* II is lower than that digested by *Msp* I due to the different sensitivities of the two isoschizomers to cytosine methylation. We should found more *Eco*R I/*Msp* I fragments than *Eco*R I/*Hpa* II fragments in MSAP profiles. However we generally observed that the number of the amplified fragments of *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I had little difference, this might be that some *Eco*R I/*Msp* I fragments were shorter than 100 bp, which run out of the gel under our electrophoretic condition.

The DNA methylation status of the treatment samples varied in the study, and both *de novo* methylation and demethylation were observed. Furthermore, the samples treated in LN showed more demethylation sites than those treated not in LN. Interestingly, 29 fragments especially appeared in the cryoprotection samples, which represented 49.7% of those various bands among the total fragments. These special fragments were not observed in the controls and the cryopreservation samples. It was suggested that the processes of cryoprotection and cryostorage in LN had impact on DNA methylation status. And it was unknown whether the variation of DNA methylation status was a component of adaptation to the environmental conditions plants encountered. In plants, the DNA methylation of promoter regions usually inhibits transcription. But methylation in coding regions does not generally affect

Table 2. MSAP-banding patterns in three groups

Banding pattern	1	2	3	No. of MSAP bands
No. 1	I	I	I	459
No. 2	II	II	II	40
No. 3	III	III	III	49
No. 4	–	II	–	15*
No. 5	III	I	III	2*
No. 6	I	I	III	3
No. 7	I	III	III	3
No. 8	I	II	II	5
No. 9	III	III	I	3
No. 10	III	–	–	4
No. 15	I	II	I	3*
No. 16	–	I	–	2*
No. 17	I	–	I	2*
No. 18	–	III	III	2
No. 19	I	III	I	2*
No. 20	–	–	I	2
No. 21	–	–	II	1
No. 22	–	–	III	1
No. 23	II	I	I	1
No. 24	II	I	II	1*
No. 25	–	I	I	3
No. 26	III	I	I	2
No. 27	I	I	II	1
No. 28	III	–	III	1
No. 29	II	–	I	1
No. 30	III	II	II	1

* refer to the specific patterns which present in the samples treated with the steps of cryopreservation except freezing in LN and thawing in 40 °C water bath. 1: control; 2: treatments except cryostorage and thawing steps; 3: treatments with whole process of cryopreservation.

Table 3. MSAP band type changes during the treatments

Band type	controls→plants treated without freeze-thawing	controls→ plants after cryopre-servation	plants treated with-out freeze-thaw ing→plants after cryopreservation
I → –	2	0	2
II → –	1	0	15
III → –	5	4	0
– → I	5	5	5
– → II	15	1	2
– → III	2	3	2
I → II	8	6	1
II → I	2	3	3
I → III	5	6	5
III → I	4	5	5
III → II	1	0	0
Total	50	33	40

I: type I band; II: type II band; III: type III band; –: absence; →: change.

gene expression[32]. In our study, DNA methylation status changed in the treatment samples but the phenotypic traits of those plants were normal, including the times of beginning to stalk and flowering, and yield-component traits etc. Further experiments are required to isolate and sequence the variable fragments, and character whether these variations could be inherited to next generation and whether the change of DNA methylation caused during cryoprotection can accumulate as the materials being cryoprotected several times.

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The authors thank other members of genetic laboratory at Henan University for their kind helps.

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Hybridized KNN and SVM for gene expression data classification[☆]

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Abstract

Support vector machine (SVM) is one of the most powerful supervised learning algorithms in gene expression analysis. The samples intermixed in another class or in the overlapped boundary region may cause the decision boundary too complex and may be harmful to improve the precise of SVM. In the present paper, hybridized k-nearest neighbor (KNN) classifiers and SVM (HKNNSVM) is proposed to deal with the problem of samples in the overlapped boundary region and to improve the performance of SVM. The first KNN is used to prune training samples and the second KNN is combined with SVM to classify the cancer samples. The proposed algorithm was used in binary and multiclass classification of gene expression data. The results were compared to those obtained by single SVM and KNN. It has been demonstrated that the proposed method is a useful tool for classification and the misclassification rate for the prediction set is reduced with samples pruning used. Compared with SVM and KNN, the misclassification rates of HKNNSVM for the datasets containing mislabeled samples were notably lower than that by SVM and KNN, which indicated that the classification performance of HKNNSVM was stable. [Life Science Journal. 2009; 6(1): 61 – 66] (ISSN: 1097 – 8135).

Keywords: support vector machine; k-nearest neighbor; gene expression data; classification

1 Introduction

Nowadays, people can obtain the expression datasets of thousands of genes simultaneously using microarray technology. One of the important fields in using these gene expression datasets is to classify and predict the diagnostic category of a sample[1,2]. Actually, precise diagnosis and classification is crucial for successful treatment of illness.

For classifying microarray data, one can use the classical liner discriminant analysis, artificial neural networks, KNN, as well as some more sophisticated machine learning methodologies including bagging, boosting and kernel methods. Among them, SVM is one of the most powerful supervised learning algorithms in gene expression analysis. SVM has been found generalization ability and useful in handling classification tasks in case of the high dimensionality and sparsity of data points.

SVM constructs an optimal hyperplane from a small set of samples near the boundary and is sensitive to these boundary samples. The samples intermixed in another class or in the overlapped boundary region may cause the decision boundary too complex and may be harmful to improve the precise of classifier. The existence of samples in the overlapped region may also increase the computation burden and decline the generalization ability of classifier. In addition, labeling a sample in some cases can be subjective and a few mislabeled samples could deeply degrade the performance of the classifier[3]. Mislabeled and troublesome learning samples may be often near the boundary and lead to a result with high error rate. Many researches[4,5] have been focused on identifying and pruning the questionable redundancy samples to improve the performance of classification.

There is increasing evidence that the ensemble classifier performs better than the individual. The combined classifiers increase not only the accuracy of the classification, but also lead to greater confidence in the result[6]. Though SVM has been found useful in handling classification tasks, it has been recognized that results of

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SVM analysis can be improved when combining with other classifiers.

In the present paper, hybridized KNN and SVM (HKNNSVM) is proposed to deal with the problem of samples in the overlapped boundary region and to improve the performance of SVM. KNN[7] is a very efficient pattern recognition method and can be easily carried out. In a statistical opinion, the error rate of a KNN classifier tends to the Bayes optimal when k and the size of sample set tend to infinity[8]. Base on these advantages, KNN is introduced into SVM to classify three gene expression datasets. We firstly used the KNN to prune training samples and then combine KNN with SVM to improve the classification. The proposed hybridized algorithm was used in binary and multiclass classification of gene expression data. The results were compared to those obtained by single SVM and KNN. In this study, linear kernel function is included in the SVM and HKNNSVM procedure, so the SVM, KNN and HKNNSVM are linear process. It has been demonstrated that the proposed method is a useful tool for classification and the classification performance is stable. It has indicated that the proposed classifier is superior to some other classifier.

The remainder of this paper is organized as follows: In Section 2, we provide the detail of our proposed procedure. Section 3 introduces three public datasets to evaluate the performance of our proposed method. The experimental resulted from our proposed method is presented, and compared with KNN and SVM method on the public datasets in section 4. Finally conclusions are drawn in section 5.

2 HKNNSVM

SVM is sensitive to these samples intermixed in another class or these boundary samples. The existence of samples in the overlapped region may be harmful to the performance of SVM. The hybridized classifier can improve the precise of classification, so hybridized of KNN classifiers and SVM is proposed to improve the performance of classification. The first KNN is used to prune training samples and the second KNN is combined with SVM to classify the cancer samples. We first yield the distances matrix which is a symmetrical matrix containing the Euclidean distance between each pair of samples. Then the K nearest neighbors for each sample are sought. In the first KNN, if the class label of training sample is same as the label of the majority of its K nearest neighbors, the training sample is reserved, whereas others are pruned. For the pruned samples set, the second KNN and SVM are applied to classify. If k nearest neighbors have all the same labels, the sample is labeled. Otherwise SVM will be applied to classify the rest sample. The hybridized KNN and SVM are described as follows.

Step1. Select relevant genes using t-test. The gene selection is an important aspect for class identification and t-test is one of the most popular gene ranking methods.

Step 2. Prune training samples by the first KNN.

Step 3. Use the second KNN to classify the remaining samples which are not pruned away from the training set. The sample is classified into the same class that its K neighbors are all in the same class, otherwise, go to the next step.

Step 4. Apply SVM to classify the rest unidentified samples. The HKNNSVM scheme is presented in Figure 1.

For test sample, the k nearest objects to it in training dataset are selected firstly, if all the k nearest objects belong to category L, then classify the test sample in L. otherwise, apply the SVM to label it.

In this study, linear kernel function is included in the SVM procedure.

3 Datasets

Three public datasets were used to test our method in this paper.

3.1 Colon data

This dataset[1] is often used for testing all kinds of classification method. It consists of 62 samples (40 tumor and 22 normal colon tissues). Gene expressions for 2000 human genes are measured using the Affymetrix technology. These data are publicly available at http://microarray.princeton.edu/oncology/affydata/index.html. For colon dataset, we constructed 50 randomly selected samples (18 normal and 32 cancer tissues) as training set and the remaining 12 samples as the prediction set.

3.2 Estrogen data

These datasets were obtained by applying the Affymetrix gene chip technology and first presented in papers by West and Spang[14,15]. The common expression matrix monitors 7129 genes in 49 breast tumor samples. In this dataset, 25 samples are labeled ER^+, the rest 24 samples are labeled ER^-. These data are retrieved from http://mgm.duke.edu/genome/dna micro/work/. Among 49 estrogen samples, 40 randomly selected samples (20

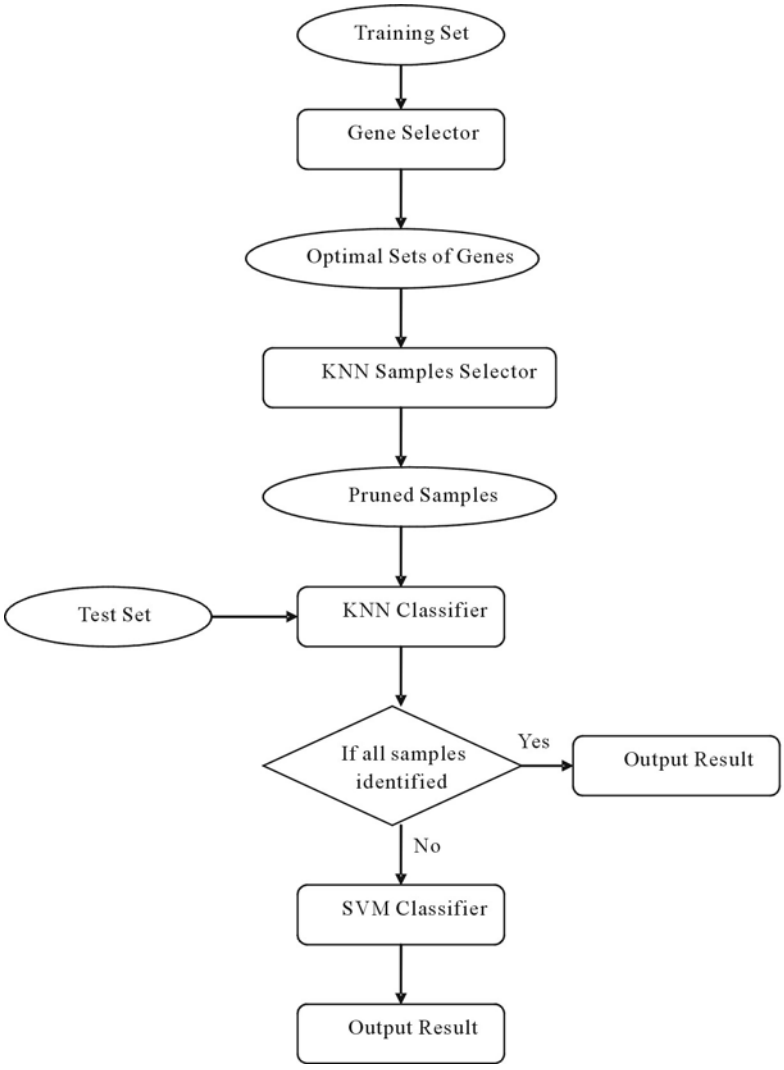

Figure 1. The chart of the HKNNSVM scheme.

are ER^+ and 20 are ER^-) were used as training set and the remaining 9 samples as the prediction set.

3.3 Acute lymphoblastic leukemia (ALL) data

ALL dataset (16) is publicly available at http://www.stjuderesearch.org/data/ALL1/all_datafiles.html. The dataset consists of expression profiles of 12625 human genes from 248 patients, there are 6 subsets: 15 BCR-ABL samples, 27 E2A-PBX1 samples, 64 Hyperdiploid > 50 chromosomes samples, 20 MLL samples, 43 T-All samples, 79 TEL-AML1 samples. For ALL dataset, we constructed 208 randomly selected samples (five-sixths for each subset) as training set and the remaining 40 samples as the prediction set.

The HKNNSVM algorithm was programmed in Matlab 6.0 and run on a personal computer (Intel Pentium processor 733MHZ 256 MB RAM).

4 Results and Discussion

4.1 The performance of classifiers for three gene expression datasets

In the present study, three publicly available datasets are used to test the performance of our method for tumor classification. The performance of classifier is measured according to an averaged classification error rates over 5-fold cross-validation. Briefly, the samples are randomly split into five data sets of approximately equal size respectively. The training data set, which is four parts of the subsets, is used to derive a classification model that is then applied to predict the remaining

subsets. The procedure should be repeated five times and the classifier is evaluated by the averaged error value over the five subsets. Because of the arbitrariness of partition of dataset, the predicted error rate of a model at each iteration is not necessarily the same. To evaluate accurately the relevance of genes subset, such 5-fold cross-validation was repeated 300 times and then averaged the error rate.

As a comparison, support vector machine and K-nearest neighbors classification methods were first utilized for these gene expression datasets. The t-test was first used on the training data to select the optimal genes. For each dataset, we select 30, 50, 100, 200 and 500 top-ranked genes using t-test statistic respectively to test the classification and then selected the best subset that obtained the lowest error. Errors for several fixed feature size for each classifier are showed in Figures 2, 3 and 4.

The misclassification rates of training and test set for each dataset by SVM and KNN were presented in Table 1.

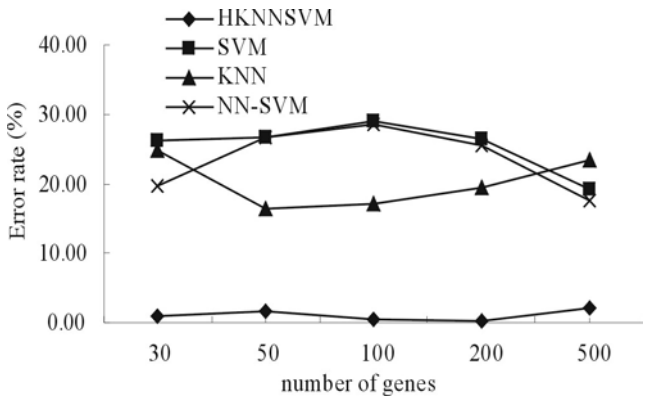

Figure 2. Error rate for colon dataset.

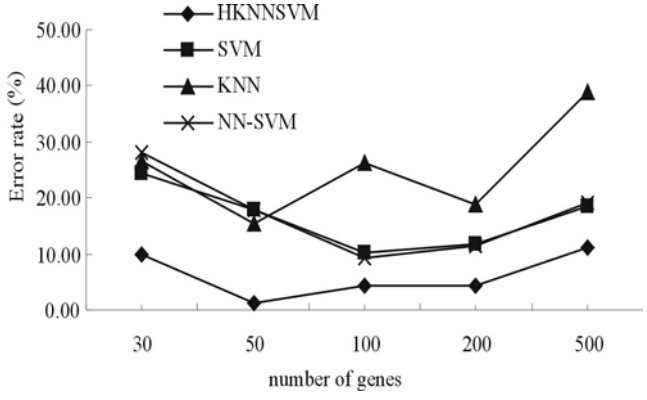

Figure 3. Error rate for Estrogen dataset.

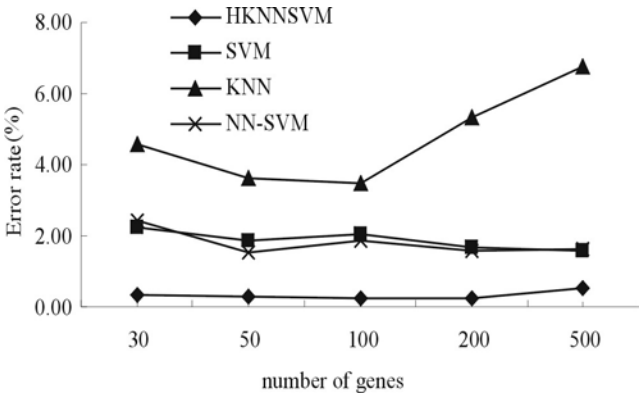

Figure 4. Error rate for ALL dataset.

For colon dataset, the optimal subset for SVM classification contains 500 genes and the misclassification rate for training set and test set were 19.20% and 12.39% respectively. Using KNN the optimal subset contains 50 genes and the misclassification rate for training set and test set were 16.40% and 12.22% respectively. For estrogen and ALL dataset, a comparison with KNN classifier shows that better results were obtained from SVM algorithm. For estrogen dataset, the optimal subset for SVM contains 100 genes. Using all 100 genes, the misclassification rate by SVM for training and prediction sets are 10.13% and 7.94% respectively. Using the optimal 500 genes, the misclassification rate for training and test sets by SVM for ALL dataset were 1.56% and 1.74% respectively.

To compare with HKNNSVM, the KNN combined with SVM classifier (KNNSVM) was also performed in which training samples was not pruned. In KNNSVM, KNN is used only for classification and not to prune training samples. The classification results of KNNSVM were also presented in Table 1. The misclassification rates for the three datasets were 11.97%, 7.85% and 1.75% respectively. A comparison of KNNSVM, SVM and KNN shows that KNN is inadequate for classification of estrogen and ALL data and better results were obtained from KNNSVM and SVM.

To further improve the classification accuracy of the prediction models, the HKNNSVM algorithm was used to evaluate the misclassification rates of the three gene expression datasets. In HKNNSVM, the first KNN is used to prune training samples and the hybridized of the second KNN and SVM is applied to classify the cancer samples. The best classification performances of the KNN are achieved when k takes values from 3 to 5. In the present work, K is selected as 5 in the first and second KNN by experience. The misclassification

rates for each dataset by HKNNSVM were also showed in Table 1. From Table 1, one can see that the misclassification rate for colon dataset was 9.75% using 200 genes. For estrogen dataset the misclassification rate was 6.33% using 50 genes. Comparing with SVM, KNN and KNNSVM, the misclassification rates by HKNNSVM are lower than that by SVM, KNN and KNNSVM, and moreover, the number of genes used by HKNNSVM was less. For ALL dataset, using the optimal 200 genes, the misclassification rate for training and test sets by HKNNSVM were 0.23% and 2.20% respectively. The misclassification rate of test set for ALL dataset by HKNNSVM is higher than that by SVM and KNNSVM. The reason for this is probably that there are only 15 samples in one of the subset of ALL dataset (BCR-ABL) and pruned training set may be too few to build classification model.

Table 1. Results of misclassification rates for three datasets

Datasets		Method			
		HKNNSVM	SVM	KNN	NN-SVM
Colon	Number of genes	200	500	50	500
	Training set	0.28%	19.20%	16.40%	17.50%
	Test set	9.75%	12.39%	12.22%	11.97%
Estrogen	Number of genes	50	100	50	100
	Training set	1.26%	10.13%	15.38%	9.25%
	Test set	6.33%	7.94%	12.19%	7.85%
ALL	Number of genes	200	500	100	500
	Training set	0.23%	1.56%	3.46%	1.61%
	Test set	2.20%	1.74%	3.69%	1.75%

4.2 The effectiveness of pruning samples

To check the effectiveness of pruning samples, ten percent of the training sample were selected randomly and mislabeled. We investigate if those mislabeled samples can be eliminated effectively by the first KNN that is used to prune training samples and the performance of classifiers affected by those mislabeled samples.

We selected and mislabeled five and four samples randomly for colon and estrogen dataset respectively. For ALL dataset, not each subset has an overlapped region with others and more than 95% overlapped region arose between BCR-ABL and Hyperdiploid > 50 samples. That is to say, the superposition of BCR-ABL and Hyperdiploid > 50 samples is the most serious one and the key to improve the performance of classifier is to classify samples of the two subsets successfully. So in this study, 6 samples of BCR-ABL and Hyperdiploid > 50 in training set were mislabeled. The percentage of pruned mislabeled samples is computed to evaluate the effective of pruning mislabeled samples. For colon dataset, 86.20% mislabeled samples were pruned. The percentage of pruned mislabeled samples are 88.25% and 97.56% for estrogen and ALL datasets respectively, indicating KNN is effective for pruning mislabeled samples.

Table 2 summarizes the classification results of datasets contained mislabeled samples. Comparing with the results of Table 1, the misclassification rates for the three datasets contained mislabeled samples were increased largely using SVM, KNN and KNNSVM classifier and a very small number of mislabeled samples could deeply degrade the performance of the classifier. Using HKNNSVM classifier, the misclassification rates are 10.78%, 9.74% and 2.03% for colon, estrogen and ALL dataset respectively. Comparing with SVM, KNN and KNNSVM, the misclassification rates by HKNNSVM were notably lower than that by SVM, KNN and KNNSVM. The introduction of pruning training samples into the hybridized of KNN and SVM improved the characteristic performance of the classifier, as the misclassification rate for the prediction set is stable and reduced with samples pruning used.

Table 2. Results of misclassification rates for dataset contained mislabeled samples

Datasets		Method			
		HKNNSVM	SVM	KNN	NN-SVM
Colon	Number of genes	200	500	50	500
	Training set	3.74%	28.7%	19.40%	26.7%
	Test set	10.78%	20.61%	12.86%	18.92%
Estrogen	Number of genes	50	100	50	100
	Training set	5.42%	23.62%	18.50%	17.13%
	Test set	9.74%	19.0%	15.41%	18.26%
ALL	Number of genes	200	500	100	500
	Training set	0.56%	2.48%	4.45%	4.16%
	Test set	2.03%	3.07%	3.53%	4.08%

5 Conclusion

In this paper, we applied the hybridized of KNN and

SVM for gene expression data classification. Our method test three public datasets and have good performance. The proposed method was used to prune the mislabeled training samples and can eliminate those samples effectively. Meanwhile the misclassification rates of our propose method not increase sharply.

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Effects of nitric oxide on salt stress tolerance in *Kosteletzkya virginica*☆

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Abstract

Salt stress is considered to be a major limiting factor for plant growth and crop productivity. Our previous study showed that exogenous application of sodium nitroprusside (60 μM SNP), a nitric oxide (NO) donor, could attenuate salt injuries in *Kosteletzkya virginica*. To further understand such protective roles of NO against salt stress, the effects of exogenous SNP on dry weight, activities of major antioxidant enzymes, proline accumulation, lipid peroxidation and distribution of sodium in *K. virginica* under salt stress were investigated. Application of SNP can increase dry weight, activities of catalase, peroxidase and superoxide dismutase, proline accumulation and decrease MDA contents in the presence of SNP under salt stress. Treatment with NaCl at 100, 200, 300 and 400 mM and SNP reduced Na^+ levels but increased K^+ levels in roots and shoots in comparison with the NaCl-treated plants. Correspondingly, the plants treated with exogenous SNP and NaCl maintained a lower ratio of $[Na^+]/[K^+]$ in NaCl-stressed plants. These data suggest that NO might confer salt tolerance in *K. virginica* by preventing both oxidative membrane damage and translocation of Na^+ from root to shoots. [Life Science Journal. 2009; 6(1): 67 – 75] (ISSN: 1097 – 8135).

Keywords: *Kosteletzkya virginica*; salt stress; NO; physiological effects

1 Introduction

Kosteletzkya virginica (*K. virginica*) is an obligate wetland species native in the southeastern US. It is a salt tolerant hardy perennial herb that performs well in salt marsh and freshwater restoration and mitigation projects as well as in storm water treatment ponds[1]. Its niche in salt marshes and above mean high tide zones foretells its tolerance for salt stress.

Leakage of electrons to the cell milieu occurs during normal electron transport in the mitochondria and chloroplasts. These leaked electrons react with O_2 during aerobic metabolism to produce activated oxygen species such as superoxide, hydrogen peroxide and hydroxyl radical[2]. These activated oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids[3]. The enzyme superoxide dismutase (SOD) converts ($O2^{-}\cdot$) into H_2O_2. Catalase (CAT) and a variety of peroxidases (POD) catalyze the breakdown of H_2O_2[4]. Although, CAT is apparently absent in the chloroplasts, H_2O_2 could be detoxified through ascorbate (Asc)-glutathione cycle in a reaction catalyzed by an As-specific peroxidase often present in high levels in this organelle[5]. Both As and glutathione were reported in millimolar concentrations within the chloroplasts[2]. The balance between the production of reactive oxygen species and the quenching activity of antioxidants becomes upset when plants are subjected to environmental salt stresses, often resulting in oxidative damage.

Nitric oxide (NO) is a small, highly diffusible gas and a ubiquitous bioactive molecule. Its chemical properties make NO a versatile signal molecule that functions through interactions with cellular targets via either redox or additive chemistry[6]. It was found to regulate the expression of mitogen activated protein (MAP) kinases[7], pathogenesis related protein (PR-1), which are proteins involved in programmed cell death and plant-pathogen

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responses[8] and can counteract other phenomena such as cell death, ion leakage, and DNA fragmentation. It was reported to counteract the toxicity of ROS generated by diquat or paraquat (PQ) in potato and rice[9,10].

In recent years there has been increasing evidence that NO is involved in many key physiological processes in plants under normal and salt stress conditions. NO can mediate plant regulators and ROS metabolism[11,12]. SOD rapidly converts $O_2^{-}\cdot$ to H_2O_2 and an oxygen molecule under an ordinary physiological condition. However, a large amount of NO may combine with $O_2^{-}\cdot$ to form peroxynitrite ($ONOO^-$), which has been reported to damage lipids, proteins and nucleic acids[13]. Nevertheless, $O_2^{-}\cdot$ and H_2O_2 are more toxic than NO and $ONOO^-$. Therefore, NO may protect cells from destruction[14]. Furthermore, in plants, NO is also used for other intercellular and intracellular signaling functions such as stomatal closure, germination. NO can promote germination of plant seeds without or in the presence of sodium chloride and reduce the detrimental effect of the salt stress on root growth efficiently[15,16]. The protective effect of NO in stressed roots may be at least partly due to the stimulation of SOD activity or direct scavenging of the superoxide anion. And NO was found to increase accumulation of ABA[17]. ABA accumulation is one of the most important responses of a plant to water stress and it plays a key role in stomatal closure and water maintenance of plants under osmotic stress.

NO has been suggested to have dual roles, either toxic or protective, depending on its environments[18]. The protective role of NO during osmotic stress or as a secondary messenger is in a dosage-dependent manner[15]. At low concentrations, the mechanism of NO in leaf water control is ABA-dependent. But at high concentrations, NO can maintain leaf water by inducing stomatal closure indendent of ABA accumulation which might act on gene expression[17].

In previous studies, it was reported that exogenous NO stimulated the expression of plasma membrane (PM) H^+-ATPase in plant under salt stress, which is involved in responses to biotic and abiotic stresses[12] and dramatically improved antioxidant capacity[19]. On the other hand, NO was found to serve as a signal for inducing salt resistance by increasing the ratio of K^+ to Na^+ in the calluses of reed[12].

There is no report on the effects of NO on antioxidative enzymes in *K. virginica* under NaCl stress, and this paper was to investigate whether NO is involved in the regulation of ROS metabolism, and whether exogenous NO can increase the halophyte *K. virginica* tolerance to salt stress by increasing the ratio of K^+ to Na^+.

2 Materials and Methods

2.1 Plant materials and salt treatments

Sterilized *K. virginica* seeds were placed in Petri dishes containing H_2O, and kept at 26 ºC. When seeds germinated, 15-day-old seedlings were cultivated in a hydroponic solution in a growth chamber (12 hours light periods, 25. 8 ºC, humidity 70%). All the measurements were carried out during the stage of three leaves. Sodium nitroprusside (SNP) was used as NO donors. Different concentrations of NaCl (0 mM, 100 mM, 200 mM, 300 mM, 400 mM) without or with SNP (0.06 mM), were added on the surface of the 1/2 Hogland. Controls were treated with 1/2 Hogland solutions. After 5 days of treatment, the samples of roots and shoots were collected, washed for 2 minutes by distilled water, and used immediately to examine dry weight, activities of CAT, POD, SOD, accumulation of proline, the content of malondialdehyde (MDA), and distribution of Na^+ and K^+ in plant.

2.2 Assay of enzyme activity

2.2.1 CAT activity. Root and leaf tissues were grounded into fine powder in liquid N_2 and then dissolved in 2 ml of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM Disoascorbic acid (AA), 2% (w/v) polyvinylpyrrolidone (PVP) and 0.05% (w/v) Triton X-100 using a chilled pestle and mortar. The homogenate was centrifuged at 10,000 × g for 10 minutes at 4 ºC and the supernatants were collected and used for the assays of CAT.

CAT activity was determined spectrophotometrically by measuring the rate of H_2O_2 disappearance at 240 nm, taking $\Delta\varepsilon$ at 240 nm as 43.6 /M·cm[20]. The reaction mixture contained 50 mM potassium phosphate (pH 7.0) and 10.5 mM H_2O_2. The reaction was run at 25 ºC for 2 minutes, after adding the enzyme extract containing 20 μg of protein and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity.

2.2.2 POD activity. The measurement of total POD activity was carried out based on the determination of guaiacol oxidation [extinction coefficient 26.6 mmol/(L·cm)] at 470 nm by H_2O_2[21]. In brief, approximately 0.50 g (fresh weight) of root or leaf tissue was homogenized on ice in 2 ml of 0.05 mM sodium phosphate buffer (pH 5.5) containing 1% PVP-40. The homogenate was centrifuged at 1,500 × g for 10 minutes at 4 ºC, and then the supernatant was diluted to 25 ml for

assays. We added 300 μl of the diluted enzyme extract into a 5-ml reaction system that contained 2.7 ml of 0.05 mM sodium phosphate buffer (pH 5.5), 1.0 ml of 2% H_2O_2, and 1.0 ml of 0.05 M guaiacol that had been incubated at 34 ºC (the optimum temperature for enzyme reaction). Immediately after the addition of enzyme extracts, the absorbency of mixture was measured at 470 nm and recorded every 30 seconds during a 150-second observation period. One unit of POD was defined as the amount of enzyme that causes a 0.01 absorbency increase at 470 nm in comparison with a blank control. In blank controls, the same amount of 0.05 M sodium phosphate buffer (pH 5.5, 300 μl) was added into the reaction system instead of the enzyme extract.

2.2.3 SOD activity. For assay of SOD, fresh roots or leaves (1 g) were homogenized in 8 mL potassium phosphate buffer (50 mM, pH 7.8) containing 0.1 mM Na_2-EDTA and 1% insoluble PVP with a chilled pestle and mortar. The homogenate was centrifuged at 20,000 × g for 20 minutes. The supernatant was collected and used for the assay of SOD following the method of Beyer and Fridovich (1987)[22]. Reaction mixture was prepared by mixing 27 ml of 50 mM potassium phosphate, pH 7.8, 1.5 ml of L-methionine (300 mg/10 ml), 1 ml of nitroblue tetrazolium salt (NBT) (14.4 mg/10 ml) and 0.75 ml of Triton X-100. Aliquots (1 ml) of this mixture were delivered into small glass tubes, followed by 20 μl of enzyme extract and 10 μl of riboflavin (4.4 mg/100 ml). The cocktail was mixed and then illuminated for 7 minutes in an aluminum foil-lined box, containing two 20 W florescent tubes. A control tube in which the sample was replaced by 20 μl of buffer was run in parallel and the absorbance A560 was measured in all tubes. The test tubes containing the reaction mixtures were exposed to light immersing the glass tubes in a cylindrical glass container three fourth filled with clean water maintained at 25 ºC, and placed in between two 20 W florescent tubes. The increase in absorbance due to formazan formation was read at 560 nm. Under the described conditions, the increase in absorbance without the enzyme extract was taken as 100% and the enzyme activity was calculated by determining the percentage inhibition per minute. About 50% inhibition was taken as equivalent to 1 unit of SOD activity. The determination of SOD activity was performed by using SOD-dependent inhibition of the reduction of NBT to purple formazan by superoxide.

2.2.4 MDA content. Standard procedures required for MDA measurement were following as Heath and Pacher[23]. Approximately 0.50 g (fresh weight) of root and leaf tissue was homogenized in 1.5 ml of 5% trichloroacetic acid (TCA, w/v). The homogenate was centrifuged at 1,500 × g for 10 minutes, and then the supernatant was diluted to 10 ml. 2 ml of the diluted extract were mixed with 2 ml of 0.67% 2-thiobarbtiuric acid (TBA, w/v). The mixture was incubated in boiled water (95 – 100 ºC) for 30 minutes, and then centrifuged at 1,500 × g for 10 minutes. Absorbencies of the aqueous phase at 450 nm, 532 nm, and 600 nm were measured respectively. MDA content in the aqueous phase was calculated according to the following formula: C (μmol/L) = 6.45 × (A532 − A600) − 0.56 × A450.

2.2.5 Estimation of proline. The concentration of proline was estimated according to the method of Bates[24]. Five grams of root or leaf tissue were homogenized with 30% sulphosalicylic acid and filtered through a Whatman No.1 filter paper. A volume of 2 ml of glacial acetic acid and 2 ml acid ninhydrin were added to 2 ml of filtrate and incubated for 1 hour in a boiling water bath followed by cooling in ice bath. About 4 ml of toluene was then added and mixed vigorously. The chromophore containing toluene was aspirated from aqueous phase and the absorbance was measured at 520 nm.

2.2.6 Distribution of Na^+/K^+. The roots, shoots and leaves of the plants were rinsed with deionized water three times, and then dried at 80 ºC to a constant weight after filtration with Whatman paper. 0.1 g dry powder samples were then extracted with 5 ml 4 M HNO_3 at 37 ºC overnight to release the free cations and centrifuged at 10,000 × g for 10 minutes. The resulting supernatants of the extracts were diluted and Na^+ and K^+ determined with a Shimadzu AA-680 atomic absorption/flame spectrophotometer.

2.27 Statistical analysis. Values presented were means ± one standard deviation (SD) of three replicates. Statistical analysis of the results was carried out according to two-sample paired t-test for means at a 0.05 probability level using SAS software.

3 Results

3.1 Shoot and root dry weights

After plants were treated for five days, they were separated into shoot and root. The shoots and roots were washed with deionized water and were then dried for dry weight determination and subsequently for analysis. The dry weight of both shoot and root of plants was slightly increased when *K. virginica* seedlings were treated with 100 and 200 mM NaCl, while slightly decreased with 300 and 400 mM NaCl stress compared to control (0 mM

NaCl). Shoot and root dry weights of plants grown under salt stress are shown in Figure 1. The dry weights of both roots and leaves were significantly increased by the NO treatments ($P < 0.05$) (Figure 1). Without salt stress, NO treatment slightly increased the dry weight of both shoots and roots.

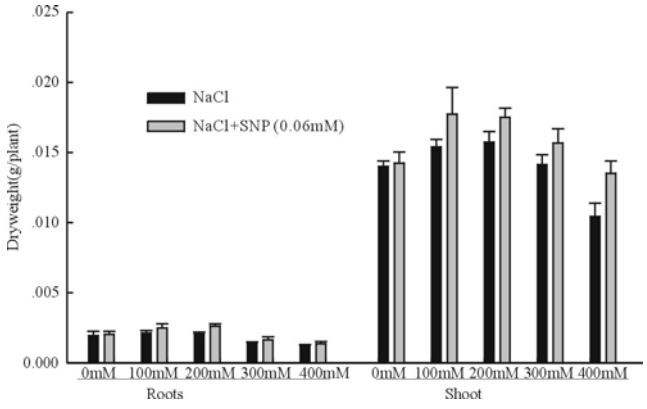

Figure 1. Effect of SNP supply on root and shoot dry weight of 5 days treatment in *K. virginica* plants grown in half Hogland solution with NaCl without or with SNP (60 μM). Data are means ± SD of three replicates. Roots: $P = 0.0265$; leaves: $P = 0.0191$.

3.2 Antioxidant enzyme activity

The activities of three antioxidative enzymes (CAT, POD and SOD) in *K. virginica* under different levels of salt treatment without or with SNP were assayed. When *K. virginica* seedlings were treated with 100, 200, 300 and 400 mM NaCl without SNP, CAT activies in leaves showed significant decrease at 200 mM NaCl while decrease roots at 100 mM NaCl in comparision with control. In contrast, plants grown at 200 mM NaCl showed significant increases in SOD, POD activities of roots and leaves compared to that grown at 0 mM NaCl .

To determine SNP effects on plant growth and antioxidant activity, *K. virginica* seedlings were also treated with 0, 100, 200, 300 and 400 mM NaCl with SNP, the results also indicated the activities of antioxidant enzymes in roots and leaves of *K. virginica* seedlings increased when treated with SNP and salt stress in comparison with salt stress treated only (Figure 2, Figure 3, Figure 4). Treatment with SNP (60 μM) resulted in remarkable increase in the activities of POD (Figure 3) in roots and leaves and SOD (Figure 4) in leaves of *K. virginica* seedlings ($P < 0.05$) and slight increase in the roots and leaves of the activities of CAT ($P > 0.05$) (Figure 2).

3.3 Content of proline

There was an increase in proline content with increase in salinity in the roots of *K. virginica* seedlings. Moreover, application of SNP exogenously enhanced this remarkable increase in roots and leaves compared with treatment of salt stress alone ($P < 0.05$) (Figure 5).

3.4 Analysis of lipid peroxidation

The content of MDA is an indicator of lipid peroxidation and oxidative damage to membrane. Figure 6 showed that salt treatment caused a significant increase in comparison with the control in MDA content whereas SNP treatment slightly inhibited the increase in MDA contents in shoots and roots of *K.virginica* seedlings

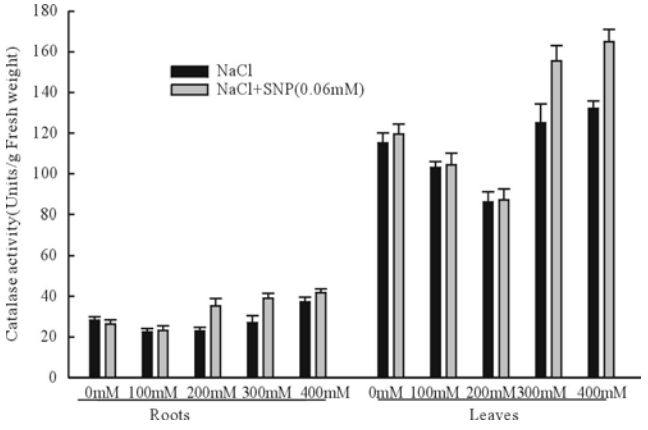

Figure 2. CAT activity (units/g fresh weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60 μM). Data are means ± SD of three replicates. Roots: $P = 0.1234$; leaves: $P = 0.1249$.

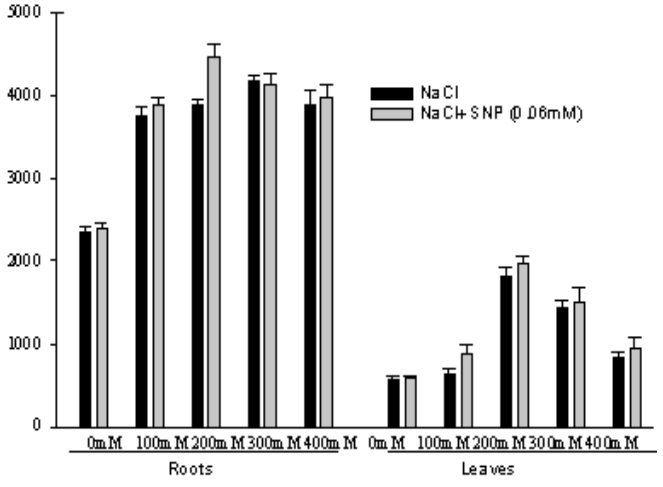

Figure 3. POD activity (units/g fresh weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60 μM). Data are means ± SD of three replicates. Roots: $P = 0.0240$; leaves: $P = 0.0348$.

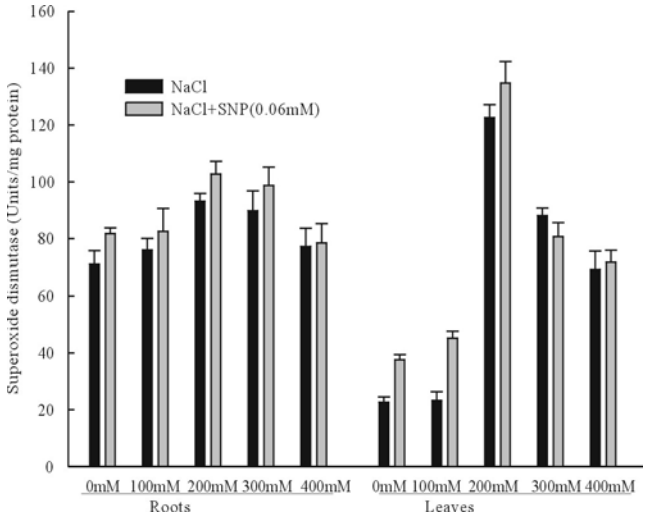

Figure 4. SOD activity (units/mg protein) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60 μM). Data are means ± SD of three replicates. Roots: $P = 0.0116$; leaves: $P = 0.1601$.

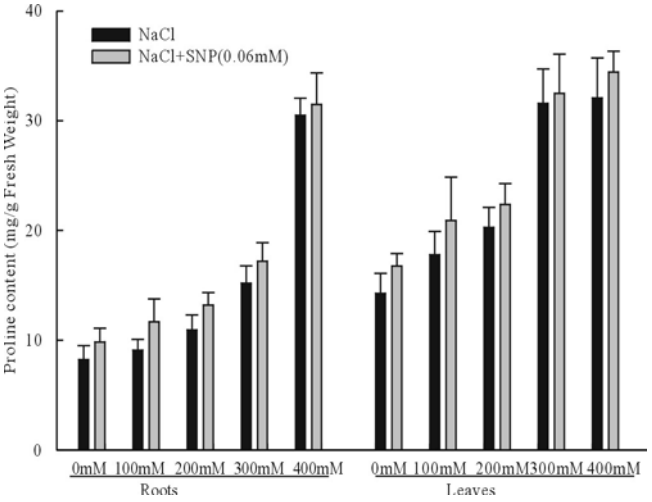

Figure 5. Proline content (mg/g frshsh weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60 μM). Data are means ± SD of three replicates. Roots: $P = 0.0024$; leaves: $P = 0.0038$.

induced by salt stress in roots and leaves ($P > 0.05$) (Figure 6).

3.5 Ratio of Na^+/K^+

Figures 7 – 9 showed the influence of salinity stress on Na^+ contents, K^+ contents and Na^+/K^+ ratio in the roots and shoots of *K. virginica* at different concentrations of NaCl (0, 100 and 200 mM) without or with SNP (60 μM). *K. virginica* plants accumulated Na^+ mainly in the roots under salinity conditions (Figure 8). Treatment with SNP significantly decreased the Na^+/K^+ ratio and Na^+ contents (Figure 7 and Figure 8), increased the K^+ contents (Figure 9) of *K. virginica* seedlings in both shoots and roots under salt stress. Maybe exogenous NO limited Na^+ absorption and transport, while promoted K^+ absorption. Our experiments showed that NO might confer salt tolerance on *K. virginica* seedlings.

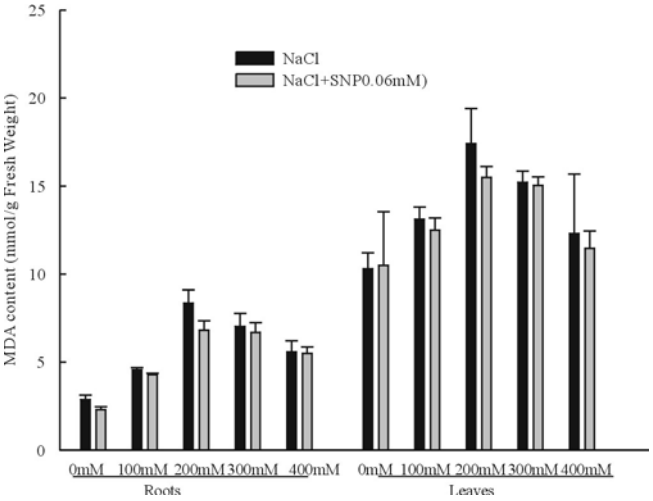

Figure 6. MDA content (mmol/g Frshsh Weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60 μM). Data are means ± SD of three replicates. Roots: $P = 0.0964$; leaves: $P = 0.1346$.

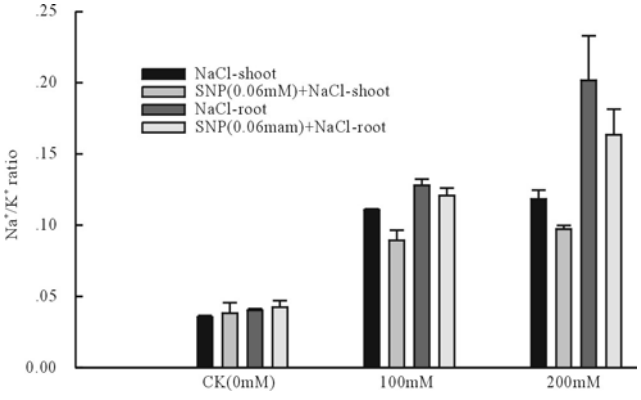

Figure 7. Effect of SNP (0.06 mM) on the Na^+/K^+ ratio of *K. virginica* seedlings under non-stress or salt stress.

4 Discussion

NO, an endogenous signaling molecule in plants, mediates responses to abiotic and biotic stresses. It was reported to be involved in the responses to drought stress[25–28], heat stress[29], disease resistance[30,31], apoptosis[32], and formation of lateral root[33]. In our

studies, the dry weights of both roots and shoots of *K. virginica* seedlings were significantly increased by exogenous NO treatment. Previous studies have indicated that salt stress induced oxidative stress, which resulted in cellular membrane injuries. Plant tolerance to salt stress should partly depend on the enhancement of antioxidant defense systems including enzymatic and non-enzymatic. Antioxidantive enzymes can protect the cell structure against the ROS generated by stress condition. In this work, a significant increase in the activities of POD, SOD in roots and leaves of *K. virginica* seedlings in response to salt stress was apparent when compared with control. Increased activities of POD, SOD play a crucial role in scavenging ROS during salinity. Interestingly, SNP can improve this role.

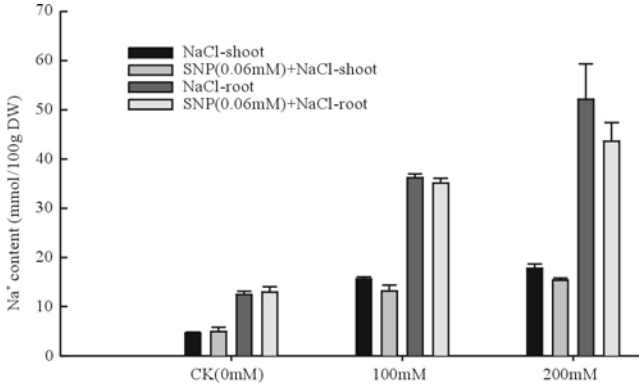

Figure 8. Effect of SNP (0.06 mM) on Na^+ contents in *K. virginica* seedlings under non-stress or salt stress.

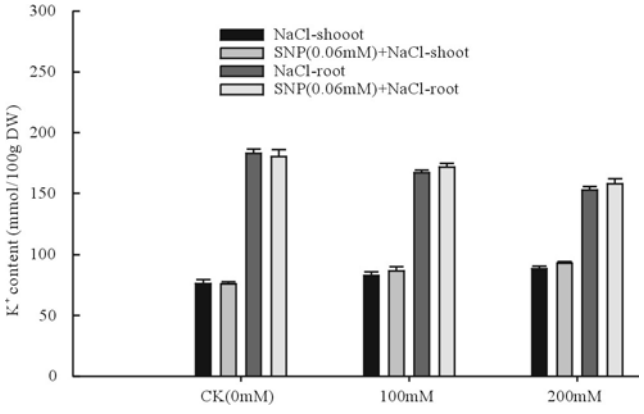

Figure 9. Effect of SNP (0.06 mM) on K^+ contents in *K. virginica* seedlings under non-stress or salt stress.

Here we demonstrate that SNP, when applied exogenously, eliminated the oxidative stress in *K. virginica* imposed by salt stress. Moreover, the activities of CAT, POD and SOD in the presence of SNP under salt stress were much higher than those under salt stress alone. This finding was in agreement with the previous study carried out by Akio Uchida (2002)[28], who found NO induced AOS (active oxygen scavenging) activities in rice under salt stress. Our results were not consistent with the results of wheat roots[8], or were unaffected as in the case of SOD in cucumber[34]. Such difference indicates that the influence of salt stress on the antioxidant enzymes is complex and related to the plant treatment time, plant tissues, plant species and genotypes.

Our experiments demonstrated that *K. virginica* seedlings accumulate proline in response to salt stress and that application of SNP exogenously enhances the increase of proline significantly under salt stress. It seems that increased level of proline has an important role in protecting enzymes involved in the antioxidant system against damaging effects of salt stress. Recently Hoque's research suggested that the concentration of proline is not high enough to adjust the osmotic potential in some plants under stress conditions[35], and that proline can mitigate the inhibition of growth of tobacco cells due to its role in antioxidant defense systems against oxidative damage of ROS. Exogenous proline increased the activities of all enzymes except MDHAR involved in NaCl-induced ASC-GSH cycle which were reduced under salt stress. It is possible that application of SNP exogenously enhanced protecting enzymes involved in the antioxidant system indirectly by the increase in proline contents.

NO may serve as a signal molecular in growth and development of plants[36,37]. However, in response to salt stress, NO can cause protection against salt stress in young rice seedlings[28] and maize seedlings[34]. A key factor limiting plant growth is excessive Na^+, a harmful mineral element not required by most plants. High Na^+ tissue content is often considered as the most critical factor responsible for salt toxicity. A possible survival strategy of plants under saline conditions was to sequester absorbed Na^+ in roots. In our work, with 100 and 200 mM NaCl treatment for 5 days, *K. virginica* plants accumulated Na^+ mainly in roots, and maintained lower $[Na^+]/[K^+]$. Treatment with SNP significantly decreased the Na^+ contents and Na^+/K^+ ratio, increased the K^+ contents of *K. virginica* seedlings in both shoots and roots under salt stress. Previous studies showed that NaCl induced a transient increase in the NO level in maize leaves. Both NO and NaCl treatment stimulated vacuolar H^+-ATPase and H^+-PPase activities, resulting in increased H^+-translocation and Na^+/H^+ exchange[38]. NO stimulated by NaCl may stimulate

plasma membrane associated PLD, which hydrolyzes membrane phospholipid and generates PA (polyamine). PA may stimulate tonoplast H^+-ATPase activity through other signaling cascades[39]. NO could be acting upstream of PA. Moreover, the downstream targets of PA have been identified in plants, including protein phosphatase and protein kinase[40]. Atnoa1 (nitric oxide synthetase gene) mutant plants displayed a greater Na^+ to K^+ ratio in shoots than wild-type plants due to enhanced accumulation of Na^+ and reduced accumulation of K^+ when exposed to NaCl. Treatment of Atnoa1 plants with SNP attenuated the NaCl-induced increase in Na^+ to K^+ ratio. NOA1-dependent NO production in Atnoa1 plants is related to its enhanced sensitivity to salt stress[41]. Our results and previous studies indicate that NO may serve as a signal in inducing salt resistance by increasing the K^+ to Na^+ ratio, which may be dependent on the increased PM H^+-ATPase activity .

Changes in protein expression and gene expression profiles of the SNP treated plants were analyzed in mung bean[42] and tobacco[7,8,43], and gene or protein expression induced by NO is involved in photosynthesis and the programmed cell death respectively. NO maybe also paradoxically act as an antioxidant and an antiapoptotic modulator that prevent cell death[44]. These cytotoxic and protective effects of NO are often concentration dependent[45]. Beside, studies on gene expression carried out by Polverari *et al*[31], who used cDNA-AFLP to profile the transcriptional changes induced by SNP and found many NO responsive genes, were previously reported to be modulated in disease-related experiments. Recently, NtGRAS1 expression was studied by treating cells with SNP, and NtGRAS1 was demonstrated to play a critical role in the transcriptional regulation of genes involved in the plant stress response[46]. In the present study, exogenous NO greatly elevated activities of antioxidant enzymes, and alleviated oxidative stress to *K. virginica* seedlings induced by salt stress. NO also decreased Na^+/K^+ ratio and enhanced accumulation of proline concentration as adaptive mechanisms. Whether or not these gene expressions of *K. virgineca* seedlings subjected to salt and SNP treatment were induced remains unclear. Here, our findings provide a perspective on protective roles of NO against salt stress, gene expression of K. virgineca seedlings subjected to salt and SNP treatment await further elucidation.

Previous studies show that NO affects mitochondrial functionality in plant cells and reduces total cell respiration due to strong inhibition of the cytochrome pathway. Nevertheless, mitochondria from all plants contains a cyanide-resistant, alternative oxidase that functions in parallel with cytochrome c oxidase as the terminal oxidase on the electron transfer chain[47]. In our experiment, whether SNP modulates mitochondrial respiration, activities of CuZn-SODs (SOD isozymes) involving in ROS metabolism, and cytochrome c of *K. virginica* seedlings under non-salt or salt stress is uncertain.

Three different enzymatic pathways and a few non-enzymatic reactions have been proposed for the generation of NO in plant roots. In addition to enzymatic NO formation by the plant, there are sources of NO in the soil and in the rhizosphere from bacterial nitrification and denitrification. NO is an uncharged lipophilic gas with a diffusion coefficient close to that of O_2 in aqueous solution. Transport of NO across membranes with lipophilic layers or that from root system to the shoot may function as a gaseous signal to send information. But NO can also lead to a high reactivity with O_2 and O_2^- and with several N compounds. In addition, dinitrogen trioxide and nitrogen dioxide are formed and represent the reactive N-nitrosating and S-nitrosating species produced during autoxidation of NO. Salinity not only induces oxidative stress but also nitrosative stress in olive leaves[48]. Salt stress caused an increase of the L-arginine-dependent production of NO, total S-nitrosothiols (RSNO) and reactive nitrogen species (RSN) occured mainly in the vascular tissue during nitrosative stress. Thus, NO in excess is toxic to higher organisms[49]. NO has either protective roles or toxic damage depending on its environments. Moreover, the effect of cytoprotective or cytotoxic action of NO on plant metabolism depends to a large extent on the local concentration of the molecule and is affected by the rate of synthesis, displacement and efficiency of removal of this reactive nitrogen species. It may be speculated that concentration and distribution of NO is important for its either positive role or negative damage in the plants. As a consequence, it is certainly worthwhile to focus further research on the formation rates of NO and relation of NO with salt tolerance.

At present, there are at least three distinct types of haemoglobins (Hbs) in plants that have been classified as symbiotic, non-symbiotic Hbs (nsHbs) and truncated Hbs. The nsHbs appear to be ubiquitous in the plant kingdom, of which class-1 Hbs have an extremely high affinity for O_2 and can be induced during stress and its activity is involved in NO degradation in a NAD(P)H-dependent manner. NO is an effective inhibitor of cytochrome oxidase in the mitochondrial electron transport chain and may further reduce cell respiration and energy prodiction. Scavenging NO of nsHbs helps

in maintaining the energy status of plant cells. The effect of nsHbs in defence against nitrosative stress is observed during treatment with NO donors[50]. In addition to Hb-based NO detoxification, some enzymes such as xanthine oxidase, glutathione peroxidase and GSNO reductase are reported to bread down NO-related species[51]. All these suggested that plants may be able to control the level of NO under different conditions. A fine-tuning of NO detoxification may exist when plants are under stress conditions.

Taken together, our study showed that exogenous application of SNP (60 μM) could attenuate salt injuries in *K. virginica* plants by acting as an efficient scavenger breaking the oxidative chain. NO imposes not only the alterations in antioxidative metabolism in *K. virginica* under NaCl stress, decrease Na^+/K^+ ratio as adaptive measures but also accumulation of osmolytes such as proline.

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Leaf extract of *Smilax schomburgkiana* exhibit selective antimicrobial properties against pathogenic microorganisms

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Abstract

The antibacterial and antifungal activities of *Smilax schomburgkiana* were investigated against pathogenic microorganisms: *S. aureus* (gram+ve), *E. coli* (gram-ve) and *C. albicans* using the stokes disc diffusion, well diffusion, streak plate methods and a dilution method. The solvent type extracts were obtained by three extractions each with hexane, CH_2Cl_2, EtOAc and CH_3CH_2OH respectively. Solvents were removed in vacuo to yield viscous oils and paste which were made up to a concentration of 0.035 g in 10 ml of the respective solvents. These were tested in varying volumes of 0.2 – 0.6 ml/plate (i.e. concentrations of 0.03 – 0.18 mg/10 ml agar). Solvents were used as control whereas ampicillin and nystatin were used as references for bacteria and fungal species respectively. The solvents had no effect on the microorganisms whereas ampicillin and nystatin inhibited microbial growth. *Smilax schomburgkiana* showed selective antimicrobial inhibitory activity at 0.18 mg/10 ml plate of medium with activity most prominent with the CH_3CH_2OH and EtOAc extracts and negligible with the hexane. This study suggests that the CH_3CH_2OH and EtOAc extracts of *Smilax schomburgkiana* can be used as herbal medicines in the control of *E. coli* and *S. aureus* and *C. albicans* induced diseases, following clinical trials. [Life Science Journal. 2009; 6(1): 76 – 83] (ISSN: 1097 – 8135).

Keywords: antimicrobial; *Smilax schomburgkiana*; stokes disc diffusion; well diffusion; streak plate; dilution method; herbal medicines

1 Introduction

This paper focuses on the antimicrobiological (antibacterial and antifungal) properties of leaves of *Smilax schomburgkiana* also known as "Dorok Waropimpla" from the coastal plain of the Guyana flora and its possible use as an herbal cream/herbal medicine. Antimicrobial properties were investigated against *S. aureus* (*SA*) (gram+ve), *E. coli* (*EC*) (gram-ve) and *C. albicans* (*CA*) strains using the stokes disc diffusion sensitivity technique, well diffusion, streak plate and a dilution method. An antimicrobial is a compound that kills or inhibits the growth of microbes such as bacteria (antibacterial activity), fungi (antifungal activity), viruses (antiviral activity) or parasites (antiparasitic activity).

There is an urgent need to revolutionalsed research in herbal medicine and isolated drug discovery considering the presence of incurable diseases such as HIV AIDS and the threat of new emerging disease such as SARS, bird flu etc. Plants are a good source of herbal medicine and natural products/phytochemicals[1–26]. Guyana has a rich biodiversified flora whose crude extracts, both organic and aqueous can be investigated for their antimicrobial activity in addition to their role as global CO_2 sinks (in the context of global warming). Also, the specified plants parts of the same species, fractionated or screened for natural products whose antimicrobial activity can also be investigated and compared with the crude extracts. Following this, clinical trials of crude extracts or fractionated natural products can lead to the formulation of an herbal plant cream or herbal medicine. A few herbal medicine shops have now been established in Guyana and the "bush" medicine man is an important figure in Guyana's culture. Plants extracts and fractionated plant extracts have been used for

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their antimicrobial properties[1–11,16–20]. Besides used as an herbal cream, following clinical trials, crude plant extracts can be chromatographed, leading to the isolation and purification of new and known bioactive natural products/phytochemicals, whose medicinal activity can also be investigated. For example, two new isopimaranes, 19-methylmalonyloxy-ent-isopimara-8,9,15-diene (5) and 19-malonyloxy-ent-isopimara-8 (9), 15-diene (6) were isolated using bioassay-guided fractionation of the CH_2Cl_2-MeOH (1 : 1) extract of the aerial part of *Calceolaria pinifolia*[21]. All compounds were assayed against *SA*, methicillin resistant *SA* (MR*SA*), *Bacillus subtilis* (*BS*) and *EC*. 4-Epi-dehydroabietinol (2) and ent-isopimara-9 (11), 15-diene-19-ol (8) were found to be active against MRSA with MIC values of 8 µg/ml and 2 µg/ml, respectively. Mechanistic studies of (8) in *BS* suggested rapid and non specific inhibition of uptake and incorporation of radiolabelled precursors into DNA, RNA, and protein consistent with membrane damaging effects in bacteria. Compound (8) didn't afford protection against an acute infection with *SA* in mice (Figure 1).

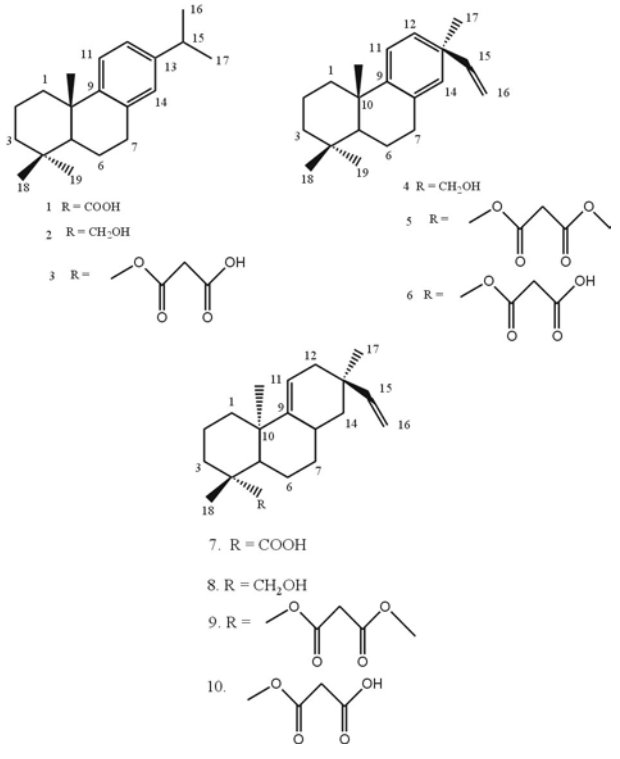

Figure 1. Structure of diterpenes (1) to (10).

Many synthetic drugs owe their discovery and potency as a result of a mimic of structures from natural products isolated from plants rather than to the creativity and imagination of contemporary organic chemists. For example, the drug taxol (a diterpenoid), first isolated from the bark of the yew tree *Taxus brevifolia* has yielded two approved drugs for breast and ovarian cancer[6].

In Guyana, there are many medicinal folklore practises but most are without scientific research. Its our scientific endeavour, to correlate antimicrobial activity of *Smilax schomburgkiana* with its folklore practices. In Guyana' s traditional medicine, a decoction of the root, and sometimes the woody stem may be mixed with kapadulla (*Doliocarpus* species), Sarsparilla (*Philodendron* species) and Devil-doer (*Strychnos* species) and used as an aphrodisiac or tonic. An infusion of the root is used to treat diseases of the urinary bladder and nervous conditions[29]. However, little is known of the antimicrobial properties of *Smilax schomburgkiana*[28]. As part of a project to investigate extracts and chromatographic fractions from plants of the Guyana's flora[16–20], for antimicrobial activity, we report here, the antimicrobial properties of *Smilax schomburgkiana*.

Smilax schomburgkiana is a high climber, often growing from a massive rootstock, stem twining, branched, terete, with a few spines and scattered blacklish tubercles, otherwise smooth and glabrous. Leaves alternate, ovate to lanceolate, acute or acuminate, rounded to cuneate at the base. Male and female flowers on separate plants in leafy racemose panicles of umbels from axils of the main stem. Its usually found in the forests of the Guianas and eastern Brazil[28]. The classification of the plant is given in Table 1.

Table 1. Scientific classification of *Smilax schomburgkiana*

Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Order	Liliales
Family	Smilacaceae
Genus	*Smilax*
Species	*Schomburgkiana*

Pathogenic microorganism investigated were *EC*, *SA* and *CA*. *EC* can cause several intestinal and extra intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia[30]. *SA* can cause furuncles (boils), carbuncles (a collection of furuncles)[31]. In infants, *SA* can cause a severe disease Staphylococcal scalded skin syndrome (SSSS). Staphylococcal endocarditis (infection of the heart valves) and pneumonia may be fatal. *CA* is a

diploid fungus (a form of yeast) and is a casual agent of opportunistic oral and genital infections in humans[32].

2 Materials and Methods

2.1 Collection of plant materials

The leaves of the above plant was collected from the University of Guyana. The deteched plant leaves were subjected to aerial drying for three weeks, removed and placed in separate conical flasks. They were then extracted with the required solvents.

2.2 Extraction

Using selective solvent extraction, the leaves were first extracted thrice in hexane over a period of five days[12–20]. Water was removed from the solvent extract by stirring over anhydrous Na_2SO_4 and extract was filtered. Solvents were removed in vacuo using a rotor vapor. The extracts was placed in sample vials and allow to evaporate. Further drying was done in a dessicator to remove residual solvents. Extracts were stored in capped vials and were weighed. The above procedure was repeated with the same leaves but with different solvents of increasing polarity: CH_2Cl_2, EtOAc, and then CH_3CH_2OH. At the end of drying process, plant extract was either viscous oils, solid or paste.

2.3 Antimicrobial activity tests

2.3.1 Making up extract solution. Approximately 0.035 g of dried crude extract of *Smilax schomburgkiana* was weighed and transferred to a 10 ml volumetric flask. The respective solvent was then added to make up the 10 ml solution, i.e 0.035 g/0.01 L.

2.3.2 Microorganisms. Pathogenic microorganisms *SA*, *EC* and *CA* were obtained from the Georgetown Public Hospital (GPH) microbiology laboratory and were stored in a refrigerator at the Food and Drug Microbiology Laboratory, Kingston.

2.3.3 Agar preparation. Two types of agar were used, nutrient agar to make up the medium for bacteria and Potato Dextrose Agar (PDA)[34,35] to make up the medium for fungi.

PDA. The potato was peeled and 100 g was measured, finely chopped and boiled to a mash in distilled water. The dextrose was measured (12.5 g) and placed in a 1 L measuring cylinder. Agar was measured (12.5 g) and added to the measuring cylinder (with the dextrose). The potato mash was stirred and strained into the cylinder. Hot distilled water was added to make up 500 ml. The contents was continuously poured and stirred until consistency was achieved. The content was then poured into a conical flask, plugged with cotton wool, over which aluminium foil was tightly wrapped. The flask was then autoclaved at 121 ºC for 24 hours. The pH range was between 6.5 – 7.0.

Nutrient agar. Nutrient agar was purchased from the International Pharmacy Association in Guyana. 14 g of nutrient agar was suspended in 500 ml of distilled water in a 1 L flask, stirred, boiled to dissolve and then autoclaved for 15 minutes at 121 ºC. The pH range was between 7.0 – 8.0. The plates were poured in a sterile environment and allowed to cool for 2 hours. Under aseptic conditions, microorganisms were streaked onto separate plates and the discs were applied with a forceps. They were labeled and placed in an incubator at 37 ºC for 24 hours and 48 hours for bacteria and fungi respectively.

2.3.4 References and control. The references were antibiotic in nature, ampicillin and nyastatin. Ampicillin was choosen as the reference for all bacterial species used, *EC* and *SA*. Nyastatin was used as the reference for the fungus, *CA*. The control experiment consists of a plate of solidifying agar onto which was inoculated pure solvent with microorganism mixed in a 1 : 1 portion[34,35].

2.3.5 Aseptic conditions. The aseptic chamber which consists of a wooden box (1 m × 1 m × 0.5 m) with a door, was cleaned with 70% ethanol and irradiated with short wave UV light (from a lamp).

2.3.6 Mother plates. These were made by culturing *CA* on PDA. A sterilized 6 mm cork borer was used to cut agar discs in the plate.

2.3.7 Disc diffusion: stokes disc diffusion sensitivity technique[34,35]. Using stokes disc diffusion sensitivity testing technique[24], an inoculum containing bacterial or yeast cells was applied onto nutrient agar plates. On each plate, a reference antibiotic was also applied. The reference antibiotic disc contained 200 mg antibiotic/ml. The discs were made by cutting discs (5 – 6 mm) from a filter paper with a perforator, placing 5 of these discs in a vial and adding 0.2 ml of each extract solution. These were left to dry. Discs were also made for the controls: ampicillin for the bacteria and nystatin for the fungus. Each disc was impregnated with the anticipated antimicrobial plant extract at appropriate concentration of 200 mg/ml using a microlitre syringe. This was then placed on a plate of sensitivity testing nutrient agar which was then incubated with the test organism: bacteria/fungi. Incubation was done at 37 ºC for 24 hours and 48 hours for the bacteria and *CA* species respectively. The antimicrobial compound diffuses from the disc into the medium. Following overnight incubation, the culture

was examined for areas of no growth around the disc (zone of inhibition). The radius of the inhibition zone was measured from the edge of the disc to the edge of the zone. The end point of inhibition is where growth starts. Larger the inhibition zone diameter, greater is the antimicrobial activities. It is anticipated through the antimicrobial activity of plant extract, no area of growth will be induced around the disc. Bacteria or fungal strains sensitive to the antimicrobial are inhibited at a distance from the disc whereas resistant strains grow up to the edge of the disc. Discs applied to the plates already streaked with bacteria and the fungus.

2.3.8 Well diffusion (diffusion plate) method. A fungus (*CA*) was inoculated into test tube containing three ml of distilled water (medium), using a flamed loop. Drops of fungus/water culture was mixed with the warm, melted, autoclaved PDA and poured into separate plates under aseptic conditions. The plates were covered and allowed to cool. As soon as the agar was partly solidified, the plates were inverted and left for 2 hours. When cooled, a well was made at the centre of the plate. The well was made by using a 6-mm cork borer or puncher that was sterilized with alcohol and flame. Plant extracts dissolve in solvent at final concentration of 0.035 g/0.01 L was pipette into the different wells in a sterilized environment at different volumes (0.2, 0.4, 0.6 ml) in separate plates, using a micro liter syringe. The four solvents (hexane, CH_2Cl_2, EtOAc and CH_3CH_2OH) at different volumes were used as control whereas nystatin dissolved in CH_2Cl_2 at same concentration with plant extract (0.035 g/0.01 L) at different volumes (0.2, 0.4, 0.6 ml) was used as the reference. The plates were labelled, covered, inverted and placed in a fume hood (no incubator was available) for 48 hours.

2.3.9 Streak plate method. Nutrient agar was prepared as described above and 10 ml was poured into plates. Plant extracts dissolved in solvent at a final concentration of 0.035 g/0.01 L were pipette into three sterilized plates under asceptic conditions at different volumes (0.2, 0.4, 0.6 ml), using a micropipette. The plates were allowed to cool and then the bacteria were streaked onto the surface of the solidified agar/plant extract medium. A flame loop was used to inoculate the bacteria from their cultures. These plates were left for 24 hours in a dessicator. The plates with inhibition were used in further experiments. A reference experiment was setup using an antibiotic (ampicillin capsule) at the same concentration as plant extracts (0.035 g/0.01 L) at different volumes (0.2, 0.4, 0.6 ml). Controls were also setup using solvents: hexane, CH_2Cl_2 and EtOAc and CH_3CH_2OH at the different volumes.

2.3.10 Luria-Bertani (LB) broth[24]. LB broth is a rich medium used to culture bacteria such as *EC* and *SA*. To make it, tryptone (10 g), yeast extract (5 g) and sodium chloride (10 g) were measured and placed in a 1 L cylinder. Distilled water was added to make up the 1 L solution and the mixture was poured and re-poured until the contents were dissolved. The pH of the solution was adjusted to 7.4 using sodium hydroxide. 3 ml each of LB broth was placed in 56 test tubes. The tubes were plugged with cotton wool foil and wrapped over each top. The tubes were placed into a beaker and autoclaved at 121 ºC for 2 hours. These tubes were used in the dilutions experiments.

2.3.11 Dilution method. This method was used to test the plant extracts for antimicrobial activities against bacteria by investigating whether there was turbity or not. Turbidity represents microbial growth, while no turbidity represents inhibition of microbes. One set of tubes containing LB was inoculated with *SA* and the second set was inoculated with *EC* using a loop, flame and alcohol. Under aseptic conditions, the plant extracts (dissolved in solvent at concentration 0.035 g/0.01 L) and showed inhibition in the streak plate were added to the one set of test tubes containing *EC* and the other set, *SA* with LB broth in differing volumes (0.2, 0.4, 0.6 ml). Two sets of four tubes each were treated with the four solvents (hexane, CH_2Cl_2 and EtOAc and CH_3CH_2OH). One set was inoculated with *SA* and the other with *EC*. Cotton wool was used to plug test tubes. The tubes were observed after 24 hours.

2.3.12 Retention factor. R_f = (Distance moved by sample)/(Distance moved by solvent front).

In general, the most polar compound has the lowest R_f value.

2.3.13 Thin layer chromatography (TLC). A baseline was drawn on the TLC plate. A spot of the plant extract was placed on the baseline with use of the pipette and allowed to dry. The plate was placed in the developing jar with the solvent. When taken out of the jar, the solvent front was drawn. The plates were then held in the iodine jar for a few seconds, shaken and taken out. They were examined under the UV/Vis lamp and the spots were circled with a pencil. The plate was further examined under UV lamp and any new spots were marked. The spots were labeled and their distances from the baseline were measured. The distance between the baseline and the solvent front was measured. The R_f values were calculated.

3 Results

Mass of dried leaves used for *Smilax schomburgkiana* species was 8.55 g respectively. These extracts were in the concentration of 0.035 g in 10 ml of solvent except for *Smilax schomburgkiana* with CH_3CH_2OH which was 0.5 g in 25 ml. This works out to 0.0003 mg/µl and 0.02 mg/µl of crude extract respectively. Ampicillin and nystatin controls were in concentration of 200 mg/ml.

3.1 Disc diffusion (Table 2)

Table 2. Antimicrobial activity of plant extract as shown by the inhibition zone diameter

Plant extracts	Reference compound (Ampicillin) (mm^2)	Area of inhibition (mm^2)			Control
		EC	*SA*	*CA*	
Hexane	28	< 5	< 5	< 5	No zone of inhibition
CH_2Cl_2	30	< 5	< 5	< 5	
EtOAc	33	23	18	20	
CH_3CH_2OH	35	24	22	23	

3.2 Well diffusion (Table 3)

3.3 Streak plate (Table 4)

Results of Table 4 were obtained from streak plate method for the bacteria's *EC* and *SA* against different volumes of dissolved plant extracts at a final concentration of 0.035 g/0.01 L and controls.

3.4 Results of dilution method (Table 5)

Table 5 showed the degree of turbidity of dissolved *Smilax schomburgkiana* extracts at concentration of 0.035 g/0.01 L at different volumes against *EC* and *SA* microbe.

3.5 TLC analysis (Table 6)

4 Discussion

All four methods, stokes disc diffusion sensitivity techniques, well diffusion, streak plate and dilution method were successful in determining *Smilax schomburgkiana* antimicrobial activities. Several trends are noted and these will be discussed first. Antimicrobial activity follow the sequence: CH_3CH_2OH extract > EtOAc extract > CH_2Cl_2 extract > hexane extract. For example, with disc diffusion method, zone of inhibition of 24 mm^2, 22 mm^2 and 23 mm^2 were obtained for the CH_3CH_2OH extract against *EC*, *SA* and *CA* in constrast to 23 mm^2, 18 mm^2 and 20 mm^2 for the EtOAc extract. For the hexane extract, zone of inhibition of < 5 mm^2 were obtained against all microbes. In a comparative method, the well diffusion, zone of inhibition of 87 mm^2 was obtained for the CH_3CH_2OH extract when the well

Table 3. Results of the well diffusion for plant extracts *Smilax schomburgkiana* against *CA*

Volume of extract (ml)	Presence of zone of inhibition/Diameter of zone of inhibition (mm^2)					
	Hexane	CH_2Cl_2	EtOAc	CH_3CH_2OH	Reference (Nystatin)	Controls
0.2	–	+/79	–	+/80	+/79	–
0.4	–	+/79	+/79	+/85	+/79	–
0.6	–	+/79	+/79	+/87	+/79	–

Table 4. Results of streak plate method for the bacteria's *EC* and *SA* against different volumes of dissolved plant extracts

Bacteria	Volume of dissolved plant extract used in ml at concentration 0.035 g/0.01 L	Inhibition or no growth of microbe (Plant extract dissolved in solvent/ Ampicillin with same concentration as dissolved plant extracts)				Reference (Ampicillin with same concentration as dissolved plant extracts)
		Hexane	CH_2Cl_2	EtOAc	CH_3CH_2OH	
EC	0.2	–/–	–/–	+/–	+/–	+
	0.4	–/–	+/–	+/–	+/–	+
	0.6	–/–	+/–	+/–	+/–	+
SA	0.2	–/–	–/–	–/–	–/–	+
	0.4	–/–	–/–	–/–	+/–	+
	0.6	–/–	–/–	–/–	+/–	+

Inhibition or no growth of microbes were represented by a positive sign (+), while the negative sign (–) represents no inhibition or growth of microbes.

Table 5. Results of the degree of turbidity of dissolved *Smilax schomburgkiana* extracts at different volumes against *EC* and *SA*

Bacteria	Volume of dissolved plant extract (ml)	Turdity (Plant extract dissolved in solvent/Ampicillin with same concentration as dissolved plant extracts)				Reference (Ampicillin with same concentration as dissolved plant extracts)
		Hexane	CH_2Cl_2	EtOAc	CH_3CH_2OH	
EC	0.2	T_3/T_3	T_3/T_3	T_2/T_3	T_0/T_3	T_0
	0.4	T_3/T_3	T_3/T_3	T_1/T_3	T_0/T_3	T_0
	0.6	T_3/T_3	T_3/T_3	T_0/T_3	T_0/T_3	T_0
SA	0.2	T_3/T_3	T_3/T_3	T_2/T_3	T_0/T_3	T_0
	0.4	T_3/T_3	T_3/T_3	T_0/T_3	T_0/T_3	T_0
	0.6	T_3/T_3	T_3/T_3	T_0/T_3	T_0/T_3	T_0

T_0 = No turbidity = Inhibition; T_1 = Lightly turbid = Moderately inhibited; T_2 = Moderately turbid = Lightly inhibited; T_3 = Very turbid = No inhibition.

Table 6. TLC analyses for all the extracts of *Smilax schomburgkiana*

Solvents	Plants extracts	No. of spots visible	R_f value
Hexane	Hexane	3	0.043
			0.173
			0.826
CH_2Cl_2	CH_2Cl_2	9	0.051
			0.077
			0.154
			0.205
			0.282
			0.436
			0.641
			0.897
			0.974
EtOAc/CH_2Cl_2 (90 : 10, v/v)	CH_3CH_2OH	8	0.048
			0.079
			0.158
			0.349
			0.381
			0.444
			0.492
			0.984

was filled with 0.6 ml of extract. For the EtOAc extract, zone of inhibition of 79 mm^2 was obtained when the volume of the well was 0.6 ml. Again, these results strongly suggest that *Smilax schomburgkiana* antimicrobial active constituents are localized in the EtOAc and CH_3CH_2OH extracts. For all methods used, the control experiments which necessitate the use of pure distilled solvent alone, rather than pure plant extract induced negative result i.e no zone of inhibition or in the case of the dilution method, turbidity in test tubes containing LB broth with bacterial microbes. The reference antibiotic ampicillin for bacteria and nyastatin for fungi induced positive results. For example, for the disc diffusion method, ampicillin induced zone of inhibition of 35 mm^2 for the CH_3CH_2OH extract whereas nystatin induced zone of inhibition of 79 mm^2 at a volume of 0.6 ml for the well diffusion method. These results suggest that *Smilax schomburgkiana* antimicrobial properties are due to the plant active constituent rather than to a solvent effect. Each solvent extracts was added in increasing volume (0.2, 0.4, 0.6 ml) to the microbial medium.

Each method display interesting results and these can be further discussed. Stokes disc diffusion indicates that the plant extract induced a larger zone of inhibition against *EC* as compared against *SA*. For example, for the EtOAc extract, zone of inhibition of 23 mm^2 and 18 mm^2 were obtained for *EC* and *SA* respectively. A similar trend was noted for the streak plate method. *EC* showed inhibition whereas *SA* showed negligible inhibition (< 5 mm^2) for the CH_2Cl_2 and EtOAc extract. However, for the CH_3CH_2OH extract extract, inhibition was observed against both *EC* and *SA*.

To investigate *CA* antimicrobial activity, the well diffusion method was used. A larger zone of inhibition was observed compared with the stokes disc diffusion method. For example, for the CH_3CH_2OH extract, a zone of inhibition of 79 mm^2 was observed at a volume of 0.6 ml. Compared with the well diffusion method, the ethanol extract at a well volume of 0.6 ml induce zone of inhibition of 87 mm^2. This difference may be ascribed to the higher sensitivity of the well diffusion method as compared with the disc diffusion method.

The streak plate method indicated selective solvent and microbial inhibition at increasing volume of from 0.2 ml to 0.6 ml. Hexane extract showed negative inhibition

against *EC* and *SA*. However, for the CH_2Cl_2 extract at a volume of 0.4 ml and EtOAc extract from a volume of 0.2 to 0.6 ml positive inhibition was observed for *EC* whereas a negative inhibition was observed for *SA*. Ethanolic extract induce inhibition against both *EC* and *SA* from 0.2 ml to 0.6 ml.

The dilution method was used to test plant extracts for antimicrobial activity against bacteria, *EC* and *SA*. The plates with inhibition from the streak plate method were used in these experiments. Results were recorded in terms of turbidity. In general, no turbidity indicates inhibition. LB broth was used as a rich medium to foster or stimulate the growth of the bacteria. *EC* and *SA* microbe induced no inhibition (very turbid mixture, T_3) for the hexane and CH_2Cl_2 extract. Complete inhibition was observed for the EtOAc extract at a volume of 0.6 ml and 0.4 ml to 0.6 ml for *EC* and *SA* respectively. CH_3CH_2OH extract at a volume 0.2 ml to 0.6 ml induce complete inhibition. The reference compound ampicillin and solvent control showed inhibition and non inhibition respectively.

TLC analysis in various solvent system for each solvent type extract revealed the presence of spots that range from three to eight (Table 6). Each spot is probably due to a pure natural product or phytochemical. Each also has a specific R_f value. The larger the R_f value, the lower the polarity of natural product/phytochemicals. The number of spots and R_f value for each spot is recorded in Table 6. For example for *Smilax schomburgkiana* EtOAc, extract using the solvent system, EtOAc/CH_2Cl_2 (90 : 10, v/v), eight spots at R_f values of 0.048, 0.079, 0.158, 0.349, 0.381, 0.444, 0.492 and 0.984 were seen.

5 Conclusion

It is clearly seen that *Smilax schomburgkiana* has antimicrobial properties which are localized primarily in the CH_3CH_2OH and EtOAc extract. However, antimicrobial activity is selective and solvent dependent with the CH_3CH_2OH extract, the most potent and hexane the least. In general, the order of antimicrobial activity follow the sequence: CH_3CH_2OH extract > EtOAc extract > CH_2Cl_2 extract > hexane extract. Thus, the CH_3CH_2OH and EtOAc extract of *Smilax schomburgkiana* can be used as the active constituent of an antimicrobial cream. Future work such as isolation and purification of bioactive constituents should target the CH_3CH_2OH and EtOAc extract of *Smilax schomburgkiana*.

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Physico-chemical analysis of treated distillery effluent irrigation responses on crop plants pea (*Pisum sativum*) and wheat (*Triticum aestivum*)

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Abstract

Physico-chemical properties of distillery effluent and their responses of different concentrations like 0%, 25%, 50%, 75% and 100% on crop plants, i.e. pea and wheat were studied under field condition. Where waste water is acidic in nature with high dissolved salts and organic load, on subsequent dilution it is serving as liquid fertilizer up to 50% effluent concentration, > 50% effluent concentration it is posing inhibitory effect on the tested crops, further work on marginal dilution are needed to substantiate the present study. [Life Science Journal. 2009; 6(1): 84 – 89] (ISSN: 1097 – 8135).

Keywords: distillery effluent; physico-chemical parameters; crop response parameters; *Pisum sativum*; *Triticum aestivum*

1 Introduction

Water pollution is prime cause of unavailability of the suitable water for irrigation purpose. Where agro-based industries are the most culprit. The total waste water produced per liter of alcohol production is around 40 to 50 liters (Vasanthy *et al*, 2006). About 40.72 million/ m^3 spent wash is generated annually from distilleries in India, considerable amount of plant nutrients are available in distillery effluent like: N = 1,660 to 4,200 mg/L, P = 225 to 3,038 mg/L, K = 9,600 mg/L, Cl = 7,238 to 42,096 mg/L, Ca = 2,050 to 7,000 mg/L, Mg = 1,715 to 2,100 mg/L, SO_4 = 240 to 425 mg/L. It also contains plant growth promoters like gibberellic acid (GA) and indol acetic acid (IAA) nearly 3246 mg/L with good fertilizers value, it contains high biological oxygen demand (BOD) and chemical oxygen demand (COD) and organic compounds like phenol, lignin, oil and grease which deteriorate the surroundings. Some compounds like endol, sketol and other sulphur compounds which not under goes effective degradation by yeast and methanogenic bacteria, are the cause of objectionable smell of distillery effluent (Murugaragavan, 2002). In water scarce situation effluent is single permanent water source for irrigation, consequently causes positive effect at lower concentrations in this relation studied has been carried out (Rani and Srivastava, 1990; Subramani, 1995; Pandey *et al*, 2007). Adverse effect on the plant and soil has been find on higher concentrations of the effluent (EC). The present study is an effort to analyze the effluent's physico-chemical parameters and their relative response on pea and wheat to speculate its suitability for irrigation purpose at their marginal dilutions.

2 Materials and Methods

2.1 Study area

Ghazipur a suburban area of district headquarters, located in the eastern Gangetic plain of the Indian sub continent at 25º 19' and 25º 54' N latitude, 83º 4' and 83º

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58' E longitude and 67.50 m above the sea level. The experiment was carried out between October 2000 to January 2001. This period of the year is characterized by mean monthly maximum temperatures between 18.6 ºC and 34.1 ºC and mean monthly minimum temperatures between 5.4 ºC and 16.7 ºC. Maximum relative humidity varied from 95% to 100% and minimum from 69% to 71%.

2.2 Selection of seeds

Seeds of wheat (*Triticum aestivum*) Var. K68 and field pea (*Pisum sativum*) Var. Swati (KFPD-24) were chosen for the experimental work both adoptive to the site of the study (Eastern U.P.) India, and recently developed and exceedingly espouse high yield varieties.

2.3 Experimental design and set up

Experiment was carried out on farmhouse near P.G. College, Ghazipur. Plot area about 24 × 15 feet, and individual replicates size was 3 × 3 feet. There were five treatments and each treatment having 3 replicates in each, means whole area has been divided in to 40 plots, each plots have separated by 20 cm deep bricks around the each plot boundary. About 100 seeds of wheat and 50 seeds of pea with uniform size for each variety were selected for each plot, the seeds were surface sterilized in 5% sodium hypochlorite solution to remove the microbial contamination, then seed were thoroughly washed with de-ionized water. The plots were prepared by proper tillage, then seeds were sown in the plots and were irrigated with 5 litter of different concentrations of the distillery effluents like 0%, 25%, 50%, 75% & 100% (here the distillery effluents are over diluted by the factory before discharge) at the interval of 5 days or as per its requirements, after 65 days plants were take-up and roots were very carefully wash to remove the soil particles along with constant safety of secondary roots.

2.4 Waste water analysis

The experiment was conducted at Department of Evironmental Science, P.G. College, Ghazipur where pH was measured with the help of pH meter (Model No. 101 E) of Electronic India, standardized with pH buffer 4, 7 and 9.2. Total dissolved solids (TDS) was estimated by evaporation method at 180 ºC, alkalinity, BOD, COD, hardness, dissolved oxygen (DO), chloride, CO_2 and all parameters were analyzed by standard procedure mentioned in APHA (1995).

2.5 Estimation of germination %, root length, shoot length and No. of secondary roots, girth of plants, leaf area and vigor index

Germination percentage was calculated by dividing the seed germinated on each day by total No. of seed taken × 100. And finally adding the total percentage. Root, and shoot length measured by normal scale of 150 mm size. Girth was measured by screw gauge; vigor index measured by Jain and Saha (1971), leaf area was measured using portable leaf area meter (Model LT 3100, LICOR).

2.6 Estimation of chlorophylls

The chlorophyll contents of primary leaves were estimated with 80% acetone with help of spectrophotometer (Hitachi, Ltd. Tokyo) (Jain and Saha, 1971).

Chlorophyll a = (0.0127) (OD_{663}) – (0.00269) (OD_{645}) gm/lit.

Chlorophyll b = (0.0229) (OD_{645}) – (0.00488) (OD_{638}) gm/lit.

Total chlorophyll = (0.0202) (OD_{645}) + (0.00802) (OD_{663}) gm/lit.

2.7 Statistical analysis

The data were subjected to mean and one way ANOVA (analysis of variance) using SPSS ver. 10 software Ducan's multiple range test performed to test the significance difference among the treatments.

3 Results

The waste water analysis results (Table 1) revealed that all the values of the different tested parameters were not compatible to the Bureau of Indian Standard (BIS). Total solid (TS), TDS, total suspended solids (TSS) values were nearly two times higher. The value of total alkalinity and total hardness were nearly one and half times higher compared to bureau of Indian standard. Value of the calcium was 182 about (7.69%) higher than BIS (169). Chloride's value was 875 about (45.83%) more than BIS (600). BOD value was 808 and COD value was 2,020 extremely higher about 2593 % and 708% than BIS values respectively. DO level was very lower < 2 than BIS value (4 – 6).

In plant responses tests, the treatments on wheat (Table 2), the entire maximum values of the plant response parameters achieved at 50% EC, except germination, chlorophyll b and root length. Maximum value of root length and chlorophyll b achieved at 25% concentration and maximum germination achieved at control condition. In case of treatments on pea (Table 3),

the entire maximum values achieved at 50% EC except germination percentage, No. of functional leaves and girth of plant. No. of functional leaf, girth of plant and germination achieved maxima at control condition.

In both plant response cases (pea and wheat), vigor achieved maxima at 50% concentration effluent and gradually decreases on further elevation on concentration (Table 4). The leaf area of wheat achieved maxima at 50% concentration and in pea (*Pisum sativum*) maxima was at control condition (Table 4). Effect of growth retardation is significant (at 0.05 level of significance) and vigorous on elevated concentrations more than 50% concentration of the effluent as evident by the aforementioned tables, while in number of functional leaves, root length and number of secondary roots (where the retardation is non significant at 0.05 level of significance).

Co-relation study revealed there is a positive correlation with increasing level of the effluent concentration up to 50% concentration of treated dis-

Table 1. Waste water analysis of treated distillery effluent

Parameters	Value	BIS
Temperatures (ºC)	38 ± 0.27	40
Color	Reddish	None
TS (mg/L)	4080 ± 0.07	2100
TDS (mg/L)	3800 ± 0.08	2100
TSS (mg/L)	280 ± 0.09	100
pH	6.5 ± 0.08	5.5 – 9
Total alkalinity (mg/L)	1437.5 ± 0.39	–
Total hardness (mg/L)	565 ± 0.05	300
Ca hardness (mg/L)	455 ± 0.05	–
Calcium (mg/L)	182 ± 0.22	169
Chloride (mg/L)	875 ± 0.56	600
DO (mg/L)	< 2	4 – 6
BOD (mg/L)	808 ± 0.76	30
COD (mg/L)	2020 ± 0.20	250
Na (mg/L)	1312 ± 0.50	–
EC	18 ± 0.9	–

Table 2. Effect of different dilution of treated distillery effluent on the growth parameters and chlorophyll content of wheat (*Triticum aestivum*)

Treatment	% germination	Shoot length (cm)	No. of functional leaf	Girth of plant (mm)	Length of root (cm)	No. of second roots	Total chlorophyll (mg/L)	Chlorophyll a (mg/L)	Chlorophyll b (mg/L)
Control	98 ± 0.11^{a}	12.3 ± 0.11^{c}	4 ± 0.57^{a}	1.23 ± 0.0096^{c}	8.85 ± 0.20^{b}	5 ± 0.57^{b}	0.006377 ± 0.037E-3^{c}	0.00230 ± 0.012E-3^{c}	0.00388 ± 0.041^{a}
25%	89 ± 0.33^{b}	14.85 ± 0.16^{b}	4 ± 1.1^{a}	1.56 ± 0.0097^{b}	12.90 ± 0.0076^{a}	6 ± 1.1^{ab}	0.00833 ± 0.02E-3^{b}	0.00241 ± 0.014E-2^{c}	0.00519 ± 0.06^{a}
50%	67 ± 0.57^{c}	19.57 ± 0.57^{a}	4 ± 0.57^{a}	2.45 ± 0.10^{a}	9.19 ± 0.0086^{b}	10 ± 2^{a}	0.00986 ± 0.067E-3^{a}	0.0271 ± 0.049E-2^{a}	0.00724 ± 0.011^{b}
75%	61 ± 0.66^{d}	15.15 ± 0.45^{b}	4 ± 0.57^{a}	2.45 ± 0.005^{a}	8.77 ± 0.0088^{b}	9 ± 1^{ab}	0.00608 ± 0.068E-3^{c}	0.0266 ± 0.062E-2^{a}	0.00388 ± 0.012^{a}
100%	30 ± 0.00^{e}	5.02 ± 0.56^{d}	3 ± 0.57^{a}	1.08 ± 0.006^{c}	8.85 ± 0.125^{b}	9 ± 0.57^{ab}	0.00550 ± 0.00^{d}	0.00835 ± 0.021E-2^{b}	0.00363 ± 0.012^{a}

Different letters in each group showed significant difference at $P < 0.05$ levels (Mean ± Stand. error).

Table 3. Effect of different dilution of treated distillery effluent on the growth parameters and chlorophyll content of pea (*Pisum sativum*)

Treatment	% germination	Shoot length (cm)	No. of functional leaf	Girth of plant (mm)	Length of root (cm)	No. of second roots	Total chlorophyll (mg/L)	Chlorophyll a (mg/L)	Chlorophyll b (mg/L)
Control	100 ± 0.0^{a}	31. 34 ± 1.1^{b}	27.42 ± 0.57^{a}	1.547 ± 0.007^{a}	7.0 ± 0.76^{a}	13 ± 0.57^{bc}	0.00694 ± 0.03E-4^{c}	0.00134 ± 0.20E-5^{b}	0.00388 ± 0.47E-5^{b}
25%	98 ± 1^{a}	30.69 ± 0.26^{b}	21.24 ± 2.6^{b}	1.40 ± 0.005^{ab}	7.5 ± 0.76^{a}	15 ± 0.66^{b}	0.00852 ± 0.02E-4^{b}	0.00281 ± 0.66E-5^{b}	0.0035 ± 0.45E-4^{b}
50%	89 ± 0.33^{b}	36.32 ± 1.7^{a}	20.00 ± 0.00^{b}	1.26 ± 0.006^{b}	7.53 ± 0.00^{a}	19 ± 0.00^{a}	0.0635 ± 0.07E-4^{a}	0.0945 ± 0.1E-3^{a}	0.0053 ± 0.15E-4^{a}
75%	70 ± 1.1^{c}	31.60 ± 0.20^{b}	19.28 ± 0.57^{b}	1.47 ± 0.0056^{a}	7.08 ± 0.39^{a}	11 ± 1^{cd}	0.00424 ± 0.08E-5^{d}	0.000138 ± 0.4E-5^{b}	0.0027 ± 0.20E-4^{c}
100%	65 ± 1.5^{d}	16.98 ± 0.13^{c}	18.4 ± 0.3^{b}	1.22 ± 0.0056^{b}	6.9 ± 0.37^{a}	10 ±1.1^{d}	0.00400 ± 0.00^{d}	0.000127 ± 0.8E-5^{b}	0.0022 ± 0.00^{c}

Different letters in each group showed significant difference at $P < 0.05$ levels (Mean ± Stand. error).

Table 4. Reading of the vigor index and the leaf area

Treatment	Vigor index		Leaf area (cm^2)	
	Pea	Wheat	Pea	Wheat
Control	3014.40 ± 3.3[a]	1306.50 ± 2.3[a]	4.86 ± 0.009[a]	27.5 ± 0.25[d]
25%	2359.60 ± 1[b]	1317.20 ± 3.8[a]	4.48 ± 0.003[b]	38.48 ± 0.002[b]
50%	1861.69 ± 3.5[bc]	1805.40 ± 6.0[a]	3.96 ± 0.09[c]	47.30 ± 0.008[a]
75%	1738.00 ± 3.2[ab]	921.10 ± 3.2[ab]	4.42 ± 0.007[b]	32.20 ± 0.41[c]
100%	439.00 ± 2.6[d]	150.60 ± 1.77[b]	2.02 ± 0.004[d]	24.7 ± 0.15[e]

tillery effluent in case of pea *(Pisum sativum*) and wheat (*Triticum aestivum*) as shown in and Figures 1 – 6 and 7 – 12, respectively.

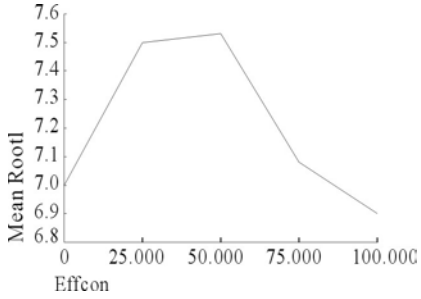

Figure 1. Co-relationship between varying levels of effluent concentrations and their responses on root length of pea.

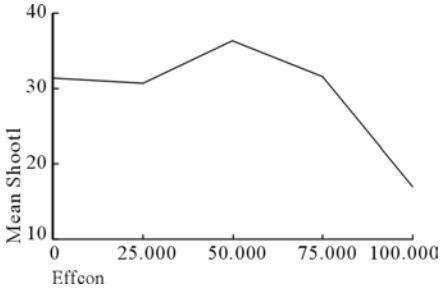

Figure 2. Co-relationship between varying levels of effluent concentrations and their responses on shoot length of pea.

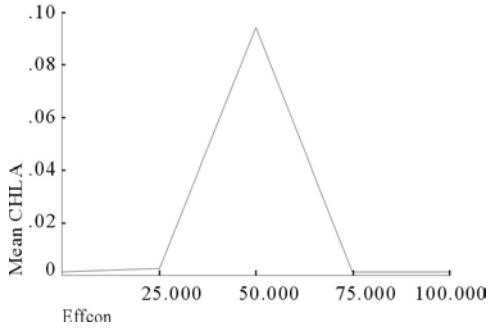

Figure 3. Co-relationship between varying levels of effluent concentrations and their responses on chlorophyll a of pea.

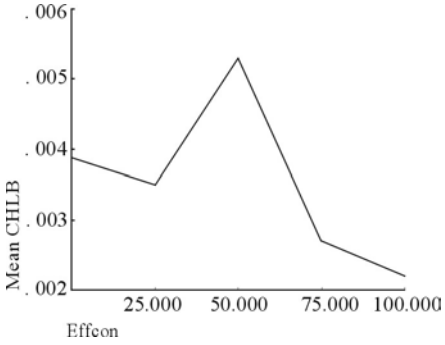

Figure 4. Co-relationship between varying levels of effluent concentrations and their responses on chlorophyll b of pea.

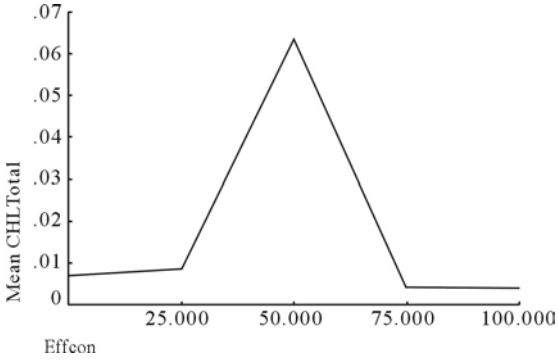

Figure 5. Co-relationship between varying levels of effluent concentrations and their responses on Total chlorophyll of pea.

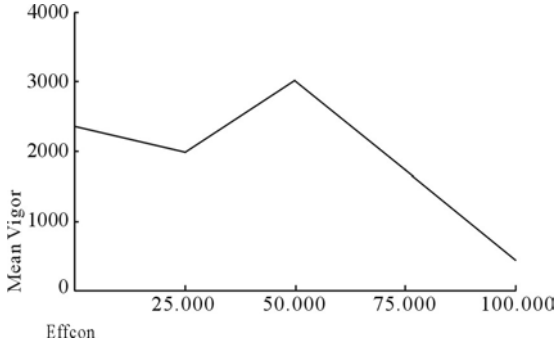

Figure 6. Co-relationship between varying levels of effluent concentrations and their responses on Vigor of pea.

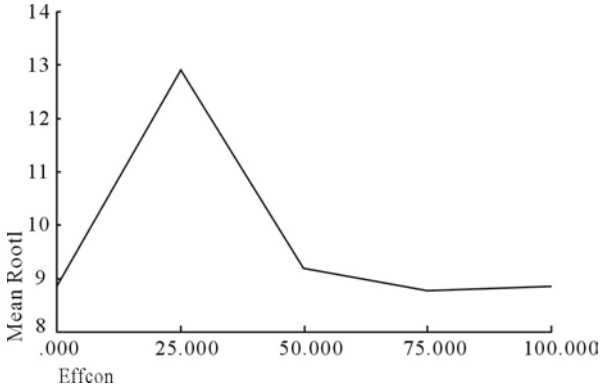

Figure 7. Co-relationship between varying levels of effluent concentrations and their responses on root length of wheat.

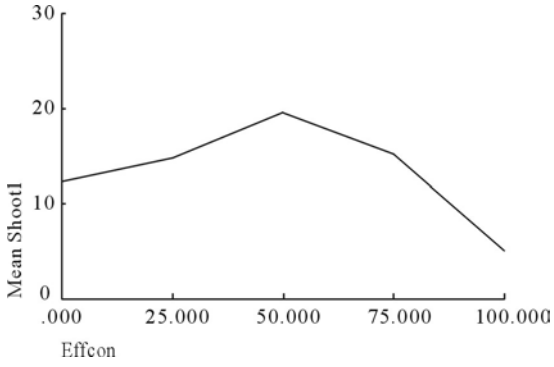

Figure 8. Co-relationship between varying levels of effluent concentrations and their responses on shoot length of wheat.

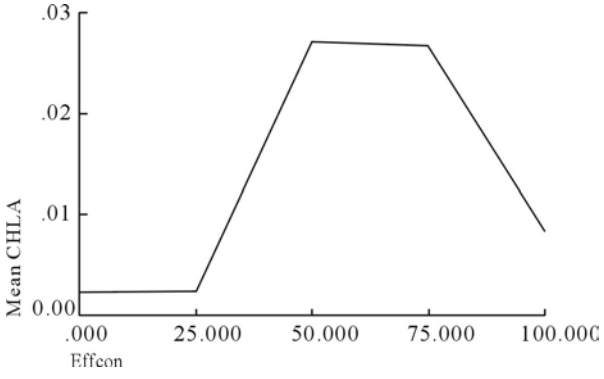

Figure 9. Co-relationship between varying levels of effluent concentrations and their responses on chlorophyll a of wheat.

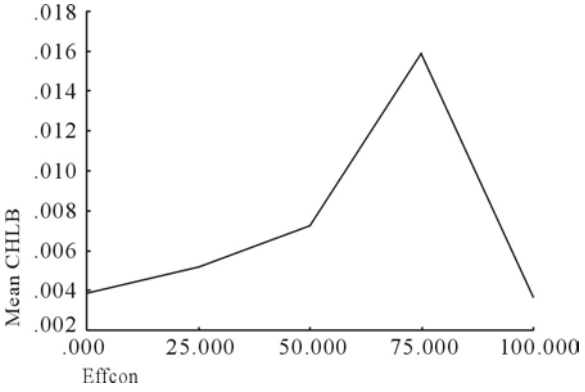

Figure 10. Co-relationship between varying levels of effluent concentrations and their responses on chlorophyll b of wheat.

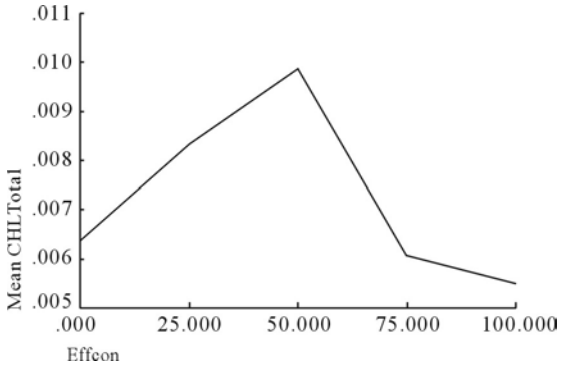

Figure 11. Co-relationship between varying levels of effluent concentrations and their responses on total chlorophyll of wheat.

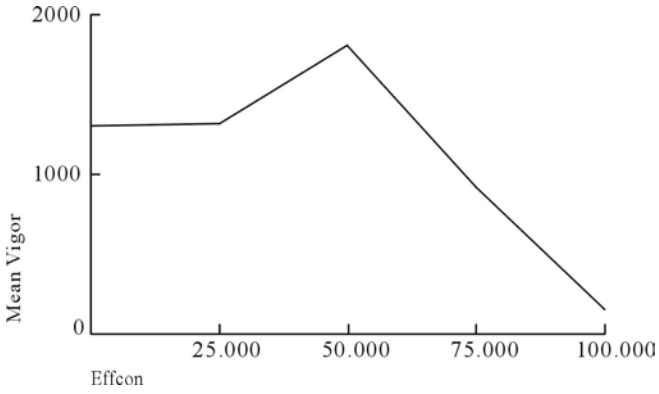

Figure 12. Co-relationship between varying levels of effluent concentrations and their responses on vigor of wheat.

4 Discussion

The extremely higher TS indicates that the effluent is very saline at the same time higher BOD and COD indicates higher organic loads, the cause of the adverse effect on the germination is the higher salinity evident by the higher EC value. The root growth at all levels (Non significant difference at 0.05 level of significance) indicates that it is not the heavy metals which has been retarding the growth parameters but the higher salinity and higher organic load, causing a cumulative retardation effect on the growth and biochemical parameters of the pea and wheat. The salinity in rhizosphere causes a higher osmotic pressure evident by

higher electrical conductivity, it causes low wall pressure and deficit of suction pressure, causes narrowing of the water balance required. The lowering water balance lowering the respiration of the plants. While increase in the organic matter content in soil increase the biodegradation and elevates the CO_2 level in rhizosphere, affecting the respiration. Three factors which governs the respiration of plants are CO_2 level in soil, water supply and temperature here CO_2 level in soil is higher, water is physiologically scarce and low temperature. These all coincide causing encumbrance in respiration, resultant is the adverse effect on physiological and biochemical parameters. The positive response on the growth parameters are at 25% to 50% concentration of the effluent is probably due to effect of effluent as liquid fertilizer (Subramani *et al*, 1995). And as gradually the concentration raises growth retardation is vigorous. It is possibly due to excess of nitrogen, phosphate, potassium, sulphate, calcium, and chloride by affecting the water absorption and other metabolic process in plants (Rani and Srivastava, 1990; Chandra *et al*, 2002). Correlations study suggested that the $\leq$ 50% concentration of distillery effluent having positive correlations with root length, shoot length, chlorophyll a, chlorophyll b, total cholorophyll, vigor, but the intra pigment dependencies are not certain for both pea and wheat plants. Still further work on marginal dilution is needed to substantiate the present study.

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Stability analysis of yield and yield related traits of rainfed rice (*Oryza sativa* L.) in an upland ultisol in Owerri

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Abstract

An experiment was conducted during the early seasons of 2004 and 2005 in Owerri to assess the stability of yield and yield components in fourteen genotypes of rice (*Oryza sativa* L.) collected from four states of Eastern Nigeria. Ten randomly selected plants were sampled in the middle row of each plot and were used for the analysis. Genotypic stability analysis was performed on yield and the most related traits to yield using mean yield coefficient of variation (C.V.). All the yield components were significant ($P = 0.05$) and positively correlated with yield in both years with the exception of number of tillers/plant in 2004 and 1000-seed weights in 2005. The genotype Fadama significantly ($P = 0.05$) produced highest grain yield as well as number of tillers/plant. Genotype NERICA 1 statistically ($P = 0.05$) produced the same grain yield with Fadama but had the highest % fertile spikelets. Genotypes independently expressed their traits in different stability groups formed. Four groups of yield estimates established were high yield and low variation, high yield and high variation, low yield and low variation and low yield and high variation. This study has shown that yield traits are complementary in action therefore selection for high and stable yield in an upland condition should include such traits as high percentage fertile spikelets, number of tillers/plant and 1000-seed weights. [Life Science Journal. 2009; 6(1): 90 – 93] (ISSN: 1097 – 8135).

Keywords: rainfed rice; *Oryza sativa* L.; stability analysis; yield parameters

1 Introduction

Rice (*Oryza sativa* L.) is speedily overtaking the regular staple crops in its consumption in Nigeria. So there is an increasing demand for rice in Nigerian markets. The performance of wetland rice is constrained by limitation in nutrient availability. Plant development and yields are severely affected by soil conditions such as moisture and soil reaction. Water requirement is larger in rice than any other annual crops. El-Hissewy *et al* (1997) reported that rice water requirement vary with variety, soil, climate and cultivation practices. Rice production under irrigation is expensive and many farmers in Nigeria cannot afford it. The most possible option would be reduction of water requirements through breeding. To achieve this, it is imperative to identify stable yield contributing characters prior to commencing the breeding programme. Rice production in Nigeria had previously been concentrated in lowland rainfed conditions. Inspite of an increased production of upland rice in Nigeria which has improved rice production. It is yet to attain self-sufficiency (Courtois, 1988). Therefore this study was undertaken to assess the stability parameters of some rainfed rice genotypes in South Eastern Nigeria in an upland condition.

2 Materials and Methods

The experiments were conducted at the Teaching and Research Farm of the Federal University of Technology, Owerri, Nigeria (5º 27' N and 7º 02' E) on an elevation of 55.10 m above sea level. The meteorological data collected from Owerri meteorological center showed that the experimental field had mean annual rainfall of 2334.40 mm and 2397.01 mm in 2004 and 2005 cropping seasons respectively. The experimental site had a mean

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annual temperature of 31 ºC and relative humidity of 89%. The particle size analysis showed that the soil had a sand value of 84.1%, silt 9.6% and clay fraction of 6.3% while the chemical soil analysis revealed a pH of 4.86, organic matter was 1.78%, total nitrogen 0.103%, 9.62 ppm Bray 2-P and exchangeable cations magnesium, potassium and calcium of 0.88, 0.52 and 0.48 cmol/ kg.

Table 1 describes the rice genotypes used in the study. The lines included five lines collected from the National Cereal Research Institute (NCRI), Baddegi, the other nine lines were collected from farmers fields in Abia, Ebonyi, Enugu and Imo States all in South Eastern Nigeria. The planting materials for the 2005 cropping season were seeds harvested from 2004 plantings.

Table1. Source, location and habitat of rice lines

S/n	Designation	Source	Habitat
1	IR-8	Abia State	Lowland
2	R Box	Imo State	Lowland
3	Mass	Ebonyi State	Lowland
4	1416	Enugu State	Lowland
5	SML	Ebonyi State	Lowland/Upland
6	Fadama	Enugu State	Lowland
7	IR-5-47	Abia State	Lowland
8	1515	Abia State	Lowland
9	BG90	Imo State	Lowland
10	NERICA2	NCRI, Badeggi	Upland
11	WAB96-1-1	NCRI, Badeggi	Upland
12	WAB450-1-B-P-160-HB	NCRI, Badeggi	Upland
13	NERICA3	NCRI, Badeggi	Upland
14	NERICA1	NCRI, Badeggi	Upland

The field was ploughed, harrowed and laid out in a complete block design with three replications. Each plot measured Im having 5 rows with spacing of 0.25 m. The rice seeds were sown directly in the field on May 25, 2004 and 5th June, 2005 by seed-drilling four seeds in each planting hole and later maintained at two stands per hole at three weeks after planting (WAP). The soil was fertilized with 100 kg NPK/ha in split-application as boost application after planting and as top dressing at booting stage. The experimental field was weeded at 5 WAP and 10 WAP. The field was secured by fencing with wire mesh to protect the rice from grass-cutter and other rodents and scare-scrows were kept for bird scaring. Post harvest operations of threshing and winnowing were done to obtain the paddy yield.

Data collected included number of days from planting to anthesis (DA), number of productive tillers per plant (PTP), plant height at flowering (PH), percentage fertile spikelets (PFS), flag leaf area (FLA), spikelets per panicle (SP), spikelets per branch of panicle (SB), a thousand seed weight (TSW) and grain yield (GY). The data were collected according to standard evaluation systems for rice SES, IRRI (1988). Ten randomly selected plants in the middle rows of each plot were used for data collection.

Combined analysis of variance for the two years was carried out for each of the yield and yield components. Genotypic correlation (rg) between yield and yield related traits was obtained from the genotypic covariance between two traits and the geometric of their genotypic variance (Obi, 1990)

$$rg = \sigma^2g(XY)/ [\sigma^2g(XX)\ \sigma^2g(YY)]^{1/2}$$

Where $\sigma^2g(XY)$ is genotypic variance of cross production of the traits X and Y, $\sigma^2g(XX)$ is the genotypic variance traits of X and $\sigma^2g(YY)$ is the genotypic variance of the trait Y.

The genotypic stability of yield and those of the two most related traits to yield were estimated by mean coefficient of variation (C.V.) approach as described by Francis and Kannerberg (1978). Using mean C.V. and grand mean of yields obtained, four groups were obtained as follows:

Group 1 is high yield and low variation, group 2 is high yield and high variation, group 3 is low yield and low variation and group 4 is low yield and high variation.

Stable genotypes for traits are those whose C.V.s are below the mean C.V. and yield higher than the grand mean yield.

3 Results and Dicussion

Significant genotypic variation (P = 0.05) was observed for all the yield and yield attributes measured (Table 2). Fadama gave the highest mean GY of 2.78 t/ha for both years and performed equally with BG90, WAB96-1-1, WAB450-1-B-P-160-HB, NERICA3, Mass and NERICA1. Genotype Fadama equally produced the highest PTP which was significantly different (P = 0.05) from those of 1R-5-47, NERICA2, WAB96-1-1, NERICA3, NERICA1 and WAB450-1-B-P-160-HB. NERICA1 had highest PFS and were statistically the same (P = 0.05) with genotypes NERICA, WAB450-1-B-P-160-HB, WAB 96-1-1 and NERICA2.

PFS and PTP partitioned the genotypes used for the study into two parallel groups with the upland rice genotypes having significantly higher PFS while the lowland ones had more tiller numbers than the upland genotypes. Genotype IR-8 produced the least GY of

0.91 t/ha and was statistically the same with some of the genotypes.

Table 3 shows the mean square of yield and yield attributes of the genotypes studied for two years. There was significant year effect on three of the yield attributes but not on GY t/ha, PTP and number of spikelets per hectare. Highly significant (P = 0.01) year effect was observed for the yield attributes with exception of number SB which varied only at P = 0.05, thus indicating genotypic variation for both years. The genotype by year (GxY) interaction was significant for half the number of yield attributes recorded and no effect on others. The variation experienced may be as a result of varietal differences.

The characters that showed non significance for GxY effect indicated stable performance inspite of differing environmental conditions for both years and thus agree with Kang (1998). Table 4 shows multiple genotypic correlation coefficients (rg) of the rice yield and related yield traits.

All the yield attributes were significantly (P = 0.05) and positively correlated with yield in 2004 with exception of PTP which negatively correlated with yield in 2004 but positively correlated with it in 2005 and was highly significant. It is thought that the environmental conditions prevailing in 2004 may have affected this trait. On the other hand, the number of SB was significant and positively correlation with yield in the first year but not in the second year. Most of the traits studied also inter-correlated positively with others. Number of SP and PFS had highest correlation values for both years and thus agrees with Lavanya *et al* (1997) who observed positive contribution of grains to high yield. Agbo and Obi (2005) reported that seed weight correlated negatively with tillers per plant and panicle per plant. In the present study PTP had no effect on TSW for the two years.

Table 2. Entry codes and yield and yield components of the upland rice genotypes used for the study

Designation	DA	PH	NPTP	PFS	FLA	GY (t/ha)	TSW (g)	NSB	NSP
IR-8	132.32	104.30	6.33	60.9	56.8	0.910	22.17	10.00	84.3
R Box	100.50	86.37	6.33	68.9	33.9	1.633	22.07	10.18	92.4
Mass	117.67	113.60	7.01	61.3	54.1	1.472	18.68	9.20	100.1
1416	113.50	92.32	6.14	54.3	50.2	1.252	19.03	9.30	78.2
SML	137.01	112.75	6.83	75.9	50.1	1.935	21.40	10.77	95.1
Fadama	99.83	80.40	7.33	70.6	38.5	2.783	21.62	10.43	102.4
IR-5-47	134.17	67.23	4.83	54.1	49.4	0.988	21.00	9.58	75.9
1515	134.71	88.25	6.02	53.9	52.6	1.507	20.63	13.20	72.5
BG90	101.17	107.43	6.17	58.0	44.8	2.185	20.08	11.67	85.3
NERICA2	74.01	80.20	5.50	79.5	57.9	1.898	22.52	10.43	77.3
WAN96-1-1	71.50	97.83	5.01	80.3	54.2	2.660	23.03	10.05	87.8
WAB450-1-B-P-160-HB	74.33	74.07	5.33	83.9	44.01	2.403	25.01	10.42	84.4
NERICA3	76.01	74.92	5.21	81.1	53.6	2.280	22.10		88.4
NERICA1	66.33	77.23	4.50	90.7	53.4	2.680	23.13		90.4
LSD	6.849	10.025	1.416	11.60	11.13	0.9056	2.303	1.44	15.47
Se	1.817	2.342	0.375	1.96	1.97	0.0598	0.213	0.365	2.29
C.V.	1.8%	2.6%	6.4%	2.8%	4.0%	3.1%	1.0%	3.5%	2.6%

LSD = Least significant difference, Se = Standard error .

Table 3. Mean square for rice grain yield and yield components in 2004 and 2005

Sources of variation	d.f	NPTP	PFS	NSB	TSW (g)	GY (t/ha)	NSP
Year (Y)	1	50.298^{**}	923.4^{**}	6.914^{*}	37.067^{**}	0.0012	5558.3^{**}
Genotypes (G)	13	$4.517^{n.s}$	939.4^{**}	$5.875^{n.s}$	16.765^{*}	2.349^{*}	$472.4^{n.s}$
GxY	13	$2.298^{n.s}$	412.2^{*}	9.690^{**}	$12.016^{n.s}$	$1.0911^{n.s}$	$456.7^{n.s}$
Error	52	1.496	100.5	1.539	3.957	0.611	178.6

*: Significant P = 0.05, **: Significant P = 0.01.

Table 4. Multiple correlation matrix of yield and yield parameters for 2004 and 2005

Year	NPTP	PFS	NSP	NSB	TSW	GY
2004	NPTP	– 0.471	– 0.631	– 0.28	– 0.383	– 0.261
	PFS		– 0.313	0.724**	0.790*	0.661*
	NSP			0.775**	– 0.365	0.607*
	NSB				0.038	0.531*
	TSW					0.521*
2005	NPTP	0.529*	0.266	0.069	– 0.392	0.719**
	PFS		0.604*	0.391	0.587	0.623*
	NSP			0.519*	– 0.189	0.982**
	NSB				0.008	0.376
	TSW					0.785**

*: Significant $P = 0.05$, **: Significant $P = 0.01$.

In Table 5, genotypes independently expressed their traits to yield in the different stability groups 1 – 4. The table shows these genotypes: NERICA1, NERICA3, WAB450-1-B-P-160-HB, and WAB 96-1-1 with 2.68, 2.28, 2.41 and 2.66 t/ha had high yields and were stable for the years under consideration.

They contrasted with genotypes Fadama, SML and BG90 which though had high yields of 2.78, 1.94, and 2.19 t/ha were unstable for the two years. Fadama also produced highest number of productive tillers but was unstable for both years. It is the opinion of this study that this might be as a result of their natural lowland adaptation since they were being tried in an upland environment. Other genotypes performed variably for the traits studied thus indicating their high contribution to yield. This agrees with Mishra and Dash (1997) and Agbo and Obi (2005) who had earlier reported similar results.

This study shows that in selecting for better GYs and adaptation under upland rice conditions, the genotypes to be grown must be stable in expression of PFS, high number of SP, high tillering ability and high number of filled grains.

4 Conclusion

The high PFS, number of PTP and TSW are highly associated with high yield in rice. Genotypes to be selected for higher yields and adaptation to upland environments should show stable PFS, high number of SP, high tillering ability as well as high number of filled grains. NERICA1, WAB450-1-B-P-160-HB and WAB96-1-1 were the genotypes that met the above requirements and are already being cultivated in upland environments. More trials are recommended in upland environments for Fadama, SML and BG90 to explore the possibility of attuning them to such environments. Some breeding works are suggested to capture the genes for high numbers of productive tillers and GY in Fadama when crossed with stable genotypes like NERICA1, WAB96-1-1 and WAB450-1-B-P-160-HB to create new genotypes.

Table 5. Grouping of rice genotypes by yield, PFS, and NPT using yield and C.V. percentage

Group	PFS	NPT	GY (t/ha)
1	NERICA1, SML, WAB450-1-B-P-160-HB, WAB96-1-1, NERICA2	BG90, 1515	NERICA1, NERICA3, WAB450-1 -B-P-160-HB, WAB96-1-1
2	WAB450-1-B-P-160-HB, Fadama	Fadama, SML, 1416, Mass, R Box	Fadama, SML, BG90
3	1515, R Box, 1416	WAB450-1-B-P-160-HB, NERICA1, IR-5-47, NERICA2	IR-8, Mass, IR-5-47, NERICA2
4	IR-5-47, IR-8, BG90, Mass	NERICA1	1416, 1515, R Box

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